

(12)

**EUROPEAN PATENT APPLICATION**

(21) Application number: **84304747.3**

(22) Date of filing: **11.07.84**

(51) Int. Cl.<sup>4</sup>: **C 07 K 7/02**  
**G 01 N 33/53, G 01 N 33/68**  
**A 61 K 37/02, C 12 N 15/00**

(30) Priority: **21.07.83 US 516136**

(43) Date of publication of application:  
**27.03.85 Bulletin 85/13**

(84) Designated Contracting States:  
**AT BE CH DE FR GB IT LI NL**

(71) Applicant: **SCRIPPS CLINIC AND RESEARCH  
FOUNDATION**  
**10666 North Torrey Pines Road**  
**La Jolla California 92037(US)**

(72) Inventor: **Sutcliffe, J. Gregor**  
**1467 Kings Cross Drive**  
**Cardiff California 92007(US)**

(74) Representative: **Grundy, Derek George Ritchie et al,**  
**CARPMAELS & RANSFORD 43, Bloomsbury Square**  
**London WC1A 2RA(GB)**

(54) **Synthetic polypeptides corresponding to portions of proteinoids translated from brain-specific mRNAs, receptors, methods and diagnostics using the same.**

(57) **Synthetic polypeptides whose sequences correspond substantially to amino acid residue sequences of at least portions of naturally occurring proteinoids translated from brain-specific mRNAs are disclosed as are receptors, methods and diagnostics that utilize those synthetic polypeptides. The synthetic polypeptides have molecular weights less than those of their corresponding proteinoids, and induce the production of antibodies that bind to the naturally occurring proteinoid, or a derivative thereof when bound to a carrier as a conjugate and are introduced into an animal.**

SYNTHETIC POLYPEPTIDES CORRESPONDING TO  
PORTIONS OF PROTEINOIDS TRANSLATED FROM  
BRAIN-SPECIFIC mRNAs, RECEPTORS, METHODS AND  
DIAGNOSTICS USING THE SAME

5 Description

Technical Field

The present invention relates to  
synthetically prepared polypeptides, and more  
specifically to synthetic polypeptides whose amino  
10 acid residue sequences correspond to the sequences of  
materials that are translated from messenger RNA  
molecules located substantially only in the brain.

Background Art

A key to understanding the function of any  
15 tissue is the biochemical characterization of the  
proteins that are specific to that tissue. The  
mammalian brain is composed of two predominate cell  
types, neurons and glia, which are organized into a  
great variety of structures. Whether the brain is  
20 viewed as one or several tissues, its organization  
involves many cells that are believed generally to  
perform the same physiological actions but at  
different places and in response to different  
signals. Accordingly, one can expect to find a large  
25 number of brain-specific proteins in a mature brain,  
including those involved in both specialized and  
general brain processes.

Exemplary of specifiable brain-specific  
proteins are: neuropeptide precursors, enzymes  
30 responsible for neurotransmitter synthesis and/or  
processing, proteins that participate in the release,  
degradation or reuptake of neurotransmitters, signal  
receptor systems and ion channels. Proteins included  
in the basic cellular structures include those  
35 peculiar to neurons (axons, dendrites and synapses)

-2-

and those involved in establishing specific cell-cell interactions. Also, there will be proteins involved in mental processes such as memory which are not at all yet understood at the cellular, let alone  
5 molecular level. Clearly, the brain is a difficult tissue to study at the molecular level due to its great complexity.

Conventional protein chemistry is unsuitable for defining rare, unique proteins or structures  
10 present in very small regions of the brains. Furthermore, approaches at the protein chemical level are dependent on having a suitable assay for each protein, which is usually not generalizable to other molecules, and which approach will clearly only be  
15 applicable to molecules that fit an existing conceptual framework, such as the hypophysiotrophic factors. Guillemin, Science, 202, 390-402 (1978).

It is presumed, however, that all of the proteins of the brain are synthesized by translation  
20 from specific messenger RNA (mRNA) molecules, and thus each brain-specific protein must have a corresponding mRNA. Thus, one approach to the study of the brain is through the transcription pattern of messenger RNA (mRNA) molecules, or by asking the  
25 question of what messenger RNA species the brain produces relative to its complete genomic potential. Estimates for the complexity of mammalian brain-specific mRNAs are very high; tens to hundreds of thousands of discrete mRNA molecules are  
30 implicated in brain function [Bantle et al., Cell, 8, 139-150 (1976) and Hastie et al., Cell, 9, 761-774 (1976).], consistent with the variety of brain-specific proteins listed above.

#### Brief Summary of the Invention

35 In accordance with the present invention, a synthetic polypeptide is prepared substantially

-3-

corresponding in amino acid residue sequence to at least a portion of the sequence of a naturally occurring proteinoid, and having a molecular weight equal to less than that of the proteinoid. The  
5 proteinoid itself contains an amino acid residue sequence that is translated from a messenger RNA present substantially only in brain cells. The messenger RNA is typically polyadenylated and is present in the cytoplasm of the brain cell tissues.  
10 The synthetic polypeptide of this invention linked to a carrier as a conjugate and introduced as that conjugate into an animal induces the production of antibodies that bind to the naturally occurring proteinoid or a derivative of that proteinoid as well  
15 as binding to the synthetic polypeptide to which those antibodies were induced.

In another embodiment of this invention, the synthetic polypeptide is introduced into an animal alone or as a conjugate bound to a carrier to induce  
20 in that animal the production of antibodies to the synthetic polypeptide. After harvesting the antibodies so induced, the harvested antibodies or idiotypic-containing polyamide portions of those antibodies, collectively referred to herein as  
25 receptors, are admixed with brain cell tissue and an indicating group to assay for the presence of an amino acid residue sequence of a naturally occurring proteinoid present in said brain tissue; the indicating group indicating the formation of an  
30 immune reaction between a proteinoid or its derivative containing the amino acid residue sequence assayed for and the receptor.

A further embodiment of the present invention is a diagnostic system for assaying for the  
35 presence of a naturally occurring amino acid residue

sequence of a proteinoid present in brain cells by the formation of an immune reaction. This system includes at least one package containing a biologically active receptor that includes an antibody or idio-  
5 type-containing polyamide portion of an antibody raised to the synthetic polypeptide of this invention or to a conjugate of that polypeptide bound to a carrier. An indicating group or label is utilized to indicate the formation of an immune  
10 reaction between the receptor and the proteinoid or derivative thereof containing the amino acid sequence assayed for when the receptor is admixed with brain tissue.

A still further embodiment of the present invention contemplates a pharmaceutical composition  
15 that contains as an active ingredient an effective amount of a synthetic polypeptide of this invention or a lyphophilic derivative of such synthetic polypeptide. The lyphophilic synthetic polypeptide  
20 derivative is capable of passage from the blood stream through the blood-brain barrier and into brain cell tissues. The pharmaceutical composition also contains a pharmaceutically acceptable diluent.

Yet another embodiment of the invention is a  
25 method for determining whether an injury has occurred to an animal's brain tissue by the formation of an immune reaction. Here, cerebrospinal fluid from an injured animal suspected of having injured brain tissue and containing a proteinoid or a proteinoid  
30 derivative released from the injured brain tissue is provided. An aliquot of that cerebrospinal fluid is admixed with effective amounts of (i) a biologically active receptor and (ii) an indicating group. The receptor is an antibody or idio-  
35 type-containing polyamide portion of an antibody raised to a

synthetic polypeptide of this invention. The admixture is then assayed for the presence of an immune reaction between a proteinoid or its derivative in the cerebrospinal fluid and the receptor. The presence of an immune reaction is indicated by the indicating group, with that immune reaction being indicative of an injury to the brain tissue containing the proteinoid or its derivative.

5 A diagnostic reagent that binds to specific brain tissue comprises yet another embodiment of the present invention. This reagent is a biologically active receptor that can be linked to an indicating group. The receptor is an antibody or  
10 idio-type-containing portion of an antibody raised to a synthetic polypeptide or conjugate of that synthetic polypeptide bound to a carrier. An immune reaction is formed when the receptor is admixed with brain cell tissue that includes a naturally occurring proteinoid or a derivative thereof. The indicating  
15 group bound to the receptor or as an exogenously supplied reagent indicates the formation of an immune reaction between the receptor and brain tissue containing the proteinoid or its derivative. In preferred practice, the proteinoid is translated from  
20 an mRNA that is present substantially only in brain cell tissue from a known type of brain cell tissue.

The present invention provides several benefits and advantages. More particularly, the invention provides useful probes through a synthetic  
25 polypeptide and the receptor molecules made therewith into the structure and function of brain cells. Thus, as one of its benefits, it is now possible to determine the presence of particular amino acid residue sequences in brain cell tissues.

Another of the benefits of the invention is the provision of a diagnostic system for assaying the presence of a naturally occurring amino acid residue sequence of a proteinoid present in brain cells.

5 Yet another benefit of the present invention is the provision of a biologically active receptor that immunologically reacts with brain cell tissues that include particular amino acid residue sequences.

10 A still further benefit of the invention is a method by which the presence of an injury to brain cell tissue may be determined.

One of the several advantages of the present invention lies in the provision of a pharmaceutical composition that contains a synthetic polypeptide of  
15 this invention present in its free form, or as a lipophilic derivative that is capable of passage from the blood stream through the blood-brain barrier and into the brain cell tissues.

20 Still further benefits and advantages of the present invention will be apparent to those skilled in the art from the detailed description and claims that follow.

#### Brief Description of Drawings

25 Figure 1 illustrates the characterization of the p1A75 complementary deoxyribonucleic acid (cDNA) of clone p1A75.

Figure 1A is a photograph of an autoradiogram illustrating Northern blot analysis of the cDNA clone p1A75 messenger ribonucleic acid (mRNA). Two micrograms each of polyadenylated  
30 messenger RNA [poly (A)<sup>+</sup> mRNA] from rat brain (B), rat liver (L), rat kidney (K), rat glioma (G), mouse neuroblastoma (N) and rat pheochromocytoma PC12 (P) were separated by electrophoresis on 1.5 percent  
35 agarose gels, blotted to nitrocellulose and

hybridized with  $^{32}\text{P}$ -labeled p1A75, or hybridized with a mixture of  $^{32}\text{P}$ -labeled p1A75 and p1B15. The positions and lengths of DNA size markers are shown at the left.

5           Figure 1B is a photograph of an  
autoradiogram illustrating Northern blot analysis of  
the regional distribution of p1A75, pO-40, and p1B15  
mRNAs. 20 Micrograms each of cytoplasmic mRNA from  
cortex (ctx), olfactory bulb (ob), caudate nucleus  
10 (cn), hippocampus (hpc), hypothalamus (hth),  
cerebellum (cb) and pons/hindbrain (hb) and 2  
micrograms of brain (total) poly (A)<sup>+</sup> mRNA were  
separated on a 1.5 percent agarose gel and the mRNAs  
specific for each clone (indicated at the right) were  
15 detected as in Figure 1A.

Figure 1C illustrates the nucleotide  
sequence of the sense strand of clone p1A75. The  
sense strand of the clone is shown having the 5'  
terminus proximal to the Eco RI cleavage site of  
20 plasmid pBR322. Also illustrated above the  
nucleotide sequence are the two putative open reading  
frames (orf 1 and orf 2) shown as the translated  
amino acid residue sequences. Three polypeptides  
(P1, P2, P3) selected for use in raising antibodies  
25 are underlined in the amino acid residue sequences.

Figure 1D illustrates a determination of the  
sense strand and open reading frames of the p1A75  
cDNA clone. Clone p1A75 is represented at the top  
showing the cDNA insert (thick line) in the same  
30 orientation as in Figure 1C in plasmid pBR322 (thin  
line). The cleavage sites of the following  
restriction enzymes are indicated; Hpa II (P), Hinf  
I (F) and Pst I. Illustrated below are both strands  
of the restriction fragments (referred to as F1,  
35 F1P1, F3 and F3P2) used to determine the sense strand



-8-

of the cDNA clone. The  $^{32}\text{P}$ -labeled 5' termini are indicated by an asterisk (\*). The positions of TGA, TAA or TAG termination codons in each of the three possible reading frames are illustrated at the bottom by darkened dots (●). Also illustrated are the two largest orfs. The position of the ATG initiation codon of orf 2 is illustrated by an open dot (o).

Figure 1E is a photograph of the autoradiogram illustrating detection of plA75 mRNA by single end-labeled restriction fragments of the plA75 cDNA insert. Two micrograms each of poly (A)<sup>+</sup> mRNA of rat brain (B) and rat liver (L) were analyzed by Northern blotting using single end-labeled restriction fragments F1P1 and F3P2 prepared as shown in Figure 1D.

Figure 2 illustrates the characterization of the protein corresponding to the translation product of clone plA75.

Figure 2A is a photograph of an autoradiogram illustrating a hybridization translation assay of clone plA75. Poly (A)<sup>+</sup> mRNA from rat brain and mRNAs which hybridized to control or plA75 filters were translated in vitro using rabbit reticulocyte lysate, and  $^{35}\text{S}$ -methionine-labeled products were fractionated by electrophoresis on 12.5 percent sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). The arrow indicates a band of approximately 25 kilodaltons (25K daltons) which appears with plA75 hybridizable mRNA but not in the control. The molecular weight estimate is based on results from comparable gels as no markers were run with these samples.

Figure 2B is a photograph of an autoradiogram illustrating a 5 to 12.5 percent gradient SDS-PAGE analysis of immunoprecipitates of

<sup>35</sup>S-methionine-labeled products from metabolically labeled rat pheochromocytoma (PC12) cells using antipeptide antibodies (either plA75 anti-P1 or control antibodies). The arrow indicates an approximately 28K dalton protein (estimated from markers run in adjacent lane) was precipitated by anti-P1 serum.

Figure 2C illustrates the alignment of the plA75 cDNA clone with its corresponding mRNA showing putative locations of coding (thick line) and non-coding regions (thin line).

Figure 3 illustrates the immunocytochemical detection of rat brain antigen with plA75 antipeptide antisera. Staining was done as described in the Materials and Methods section, hereinafter. All primary antibodies were used at a final dilution of 1:500. The same anti-P1 pattern was observed at dilutions up to 1:20,000.

Figure 3A is a photomicrograph illustrating a group of neurons in the paraventricular nucleus of the hypothalamus that show intense staining using anti-P1 antisera. The bar equals 50 microns.

Figure 3B is an enlarged view of the cells in Figure 3A showing cytoplasmic granules.

Figure 3C is a photomicrograph of neurons and dendritic processes in the subiculum stained using anti-P1 antibodies and showing frequent concentrations of immunoreactivity in the dendritic pole of the cells (arrows). The bar equals 50 microns.

Figure 3D is a photomicrograph illustrating hypothalamic cell bodies stained using anti-P2 antibodies. A stained varicose fiber is also indicated. The bar equals 50 microns.

Figure 4 illustrates the characterization of clone PlB236.

Figure 4A is a photograph of the autoradiogram illustrating Northern blot analysis of the tissue distribution of plB236 mRNA. Details are as described in Figure 1A.

5           Figure 4B is a photograph of an autoradiogram illustrating Northern blot analysis of the regional distribution of plB236 mRNA and plB15 mRNA in rat brain. Details are as described in Figure 1B.

10           Figure 4C illustrates the nucleotide sequence of the plB236 cDNA insert showing the long open reading frame and the positions of synthesized polypeptides P4, P5, P6 and P7. The 5' terminus is proximal to the Eco RI cleavage site of plasmid  
15 pBR322. Details are as described in Figure 1C.

Figure 4D illustrates the determination of the sense strand and open reading frame of clone plB236. Details are as described in Figure 1B. R is an abbreviation for restriction endonuclease Rsa I.

20           Figure 4E is a photograph of an autoradiogram illustrating detection of plB236 mRNA by single end-labeled restriction fragments prepared as indicated in Figure 4D. Details are as described in Figure 1E.

25           Figure 5 illustrates the plB236 open reading frame. Shown are the positions of potential polypeptide processing sites at pairs of basic amino acids (K=lysine, R=arginine); the positions of the synthesized polypeptides P4, P5, P6 and P7 (the  
30 numbers of the amino acids in each are shown underneath); and the position of the carboxy-terminal glycine (G) which may be involved in the amidation of polypeptide P6.

35           Figure 6 illustrates the immunocytochemical detection of rat brain antigen with plB236

antipeptide antibodies. In Figures 6A-6C and 6E-6Ha, all sections are also counterstained with Richardson's stain.

Figure 6A is a photomicrograph illustrating a low power view of cerebellum cells stained with anti-P5 antiserum showing fiber tracts: molecular (M), Purkinje cell layer (P), granule cell layer (G) and white matter (WM). The bar equals 500 microns.

Figure 6B is a photomicrograph illustrating an enlarged view of cerebellum cells showing the region boxed in Figure 6A. Radial and tangential fibers in the molecular layer are indicated by arrows. Other details are as in Figure 6A. The bar equals 25 microns.

Figure 6C is a photomicrograph illustrating fibers in the CA<sub>3</sub> region of the hippocampus stained using anti-P5 antiserum. Abbreviations: molecular layer (M) and pyramidal cell layer (P). The bar equals 50 microns.

Figure 6D is a photomicrograph illustrating a similar view as in Figure 6C but with anti-P5 antibody preabsorbed with polypeptide P5. Unreactive neurons are counterstained with cresyl violet. The bar equals 50 microns.

Figure 6E is a photomicrograph illustrating fibers in the cingulate cortex stained using anti-P5. Tangential fibers in layer 1 (indicated by a vertical bar) and radial fibers in deeper layers are shown. The horizontal bar in the lower left corner equals 50 microns.

Figure 6F is a photomicrograph illustrating fibers in cingulate cortex stained using anti-P6 antibodies. The region of cortex shown is deeper than the view in Figure 6E. The bar equals 50 microns.

-12-

Figure 6G is a photomicrograph illustrating staining in deep cerebellar nuclei using anti-P5 antibodies and showing intense peripheral staining of large cell bodies. The bar equals 50 microns.

5        Figure 6H is a photomicrograph illustrating a low power view of cell bodies stained using anti-P5 antibodies from the brain stem of a colchicine-treated rat. The bar equals 100 microns. Insert  
10       Figure 6Ha is a photomicrograph illustrating an enlarged view of the cell bodies in Figure 6H. The bar equals 25 microns.

Figure 7 illustrates the characterization of clone 1B208.

15       Figure 7A is a photograph of the autoradiogram illustrating Northern blot analysis of the tissue distribution of plB208 mRNAs. Details are as described in Figure 1A.

20       Figure 7B is a photograph of an autoradiogram illustrating Northern blot analysis of the regional distribution of plB208 mRNAs and plB15 mRNA in rat brain. Details are as described in Figure 1B.

25       Figure 7C illustrates the nucleotide sequence of the sense strand of the plB208 cDNA insert showing the translated open reading frame. The 3' terminal end of the insert sequence is proximal to ECO RI cleavage site of plasmid pBR322. Details are as described in Figure 1C.

30       Figure 7D illustrates the determination of the sense strand and open reading frame of clone 1B208. Details are as described in Figure 1D. Restriction endonuclease abbreviations are designated as follows: Sau 3A (S) and Ava II (V).

35       Figure 7E is a photograph of an autoradiogram illustrating detection of plB208 mRNAs

by single end-labeled restriction fragments prepared as indicated in Figure 7D. Fragment VIS1 was slightly contaminated with fragment VIS2 producing some detection of the larger p1B208 mRNA. Details are as described in Figure 1E.

Figure 8 illustrates the characterization of clone p0-40.

Figure 8A is a photograph of an autoradiograph illustrating Northern blot analysis of the tissue distribution of p0-40 mRNA. Details are as described in Figure 1A.

Figure 8B illustrates the nucleotide sequence of the sense strand of the p0-40 cDNA clone insert. The 5' terminus is proximal to the Eco RI cleavage site of plasmid pBR322. Details are as described in Figure 1B.

Figure 8C illustrates the possible open reading frames of p0-40. The sense strand of the cDNA insert (thick line) is represented, at the top of the Figure. Below, in the three possible reading frames of the sense strand, the positions of initiation codons are indicated by open dots (o) and termination codons are indicated by darkened dots (●). Also illustrated are the positions and lengths (in triplets) of the largest orf in each frame.

#### Detailed Description of the Invention

The present invention contemplates synthetic polypeptides, as well as products and methods obtained from their use. The synthetic polypeptides of this invention have amino acid residue sequences that correspond to at least a portion of the sequence of a naturally occurring proteinoid and have a molecular weight equal to less than that of the proteinoid.

The proteinoid amino acid residue sequence to which the sequence of the synthetic polypeptide corresponds is translated from a messenger RNA that is present substantially only in brain and/or spinal  
5 cord cells. Since the brain and spinal cord are so intimately related, these mRNA molecules are referred to herein as being brain-specific or as being present substantially only in the brain with the understanding that they may also be present in the  
10 spinal cord. Thus, the naturally occurring proteinoids, proteins and polypeptides having amino acid residue sequences corresponding to those of the synthetic polypeptides of this invention are themselves manufactured substantially only in brain  
15 cell tissues. This is in contradistinction to materials such as somatostatin, thyrotropin-releasing factor (TRF), luteinizing hormone-releasing factor (LRF), the endorphins and enkephalins, or the several other small polypeptides such as bombestin, caerulein  
20 or physalamine that have been found to occur in the brain as well as in other tissues of the animal body. [Guillemin, Science 202, 390-402 (1978).]

Definitions for several words and phrases used herein are given immediately below followed by a  
25 general discussion of the methods useful in this invention which itself is followed by a discussion of specific embodiments.

Proteinoid--The term "proteinoid" is used herein to mean the direct polypeptide translation  
30 product of a messenger RNA, and may have a molecular weight of from about 600 through about 1,000,000, and possibly larger. The proteinoid may be a single protein or a fusion product of (a) a plurality of proteins that is subsequently processed by the brain  
35 cell into a plurality of cellularly useful proteins,

(b) a fusion product of a plurality of polypeptides that is subsequently processed by the brain into a plurality of cellularly useful polypeptides, or (c) a fusion product of one or more proteins with one or more polypeptides that is subsequently processed into cellularly useful materials.

Proteinoid derivative--The phrase "proteinoid derivative" is used herein to mean the polypeptide-containing material that results from cellular processing of a proteinoid. A proteinoid derivative may therefore be a protein cleaved by cellular action from a fusion product containing a plurality of proteins, a polypeptide containing about six to about eighty amino acid residues that has neuroactivity, or the like. In addition, a proteinoid derivative may be a glycosylation product of the whole or a smaller proteinoid, or the product of a transamidification-deletion reaction or the like.

Immune reaction--The phrase "immune reaction" is used herein to mean the binding of a ligand with its receptor and includes the binding between an antigen (ligand) with an antibody (receptor) as well as between an antigen and an idiotype-containing polyamide portion of an antibody (receptor).

Corresponds substantially--The phrase "corresponds substantially" as used herein in conjunction with amino acid residue sequences means that the amino acid residue sequence of a first polypeptide is sufficiently similar to the amino acid residue sequence contained in a second polypeptide so that antibodies to the first polypeptide (e.g. synthetic polypeptide) form an immune reaction with the second polypeptide (e.g. proteinoid or derivative) when admixed in an aqueous medium. The



preparation of such polypeptides and antibodies are discussed hereinafter in the Materials and Methods Section.

5 The epitope-containing amino acid residue sequence portions of the above two polypeptides, e.g. synthetic polypeptide and proteinoid, are most preferably identical, but conservative changes in amino acid residues and deletions or additions of amino acid residues within the epitope may be made  
 10 and still permit the cross-reaction of an antibody to the first polypeptide with the second polypeptide, as is known. Conservative amino acid residue changes are well known, and include exchanges of residues such as between lysine and arginine, between aspartic  
 15 acid and glutamic acid, between leucine and isoleucine, and the like.

The full names for individual amino acid residues are used herein as are the well-known three-letter abbreviations and one-letter symbols.  
 20 The Table of Correspondence, below, provides the full name as well as the abbreviation and symbols for each amino acid residue named herein.

Table of Correspondence

		Three-letter	One-letter
25	<u>Amino acid</u>	<u>abbreviation</u>	<u>symbol</u>
	Alanine	Ala	A
	Arginine	Arg	R
	Asparagine	Asn	N
	Aspartic acid	Asp	D
30	Asparagine or aspartic acid	Asx	B
	Cysteine	Cys	C
	Glutamine	Gln	Q
	Glutamic acid	Glu	E
	Glutamine or glutamic acid	Glx	Z
35	Glycine	— Gly	G

-17-

	Histidine	His	H
	Isoleucine	Ile	I
	Leucine	Leu	L
	Lysine	Lys	K
5	Methionine	Met	M
	Phenylalanine	Phe	F
	Proline	Pro	P
	Serine	Ser	S
	Threonine	Thr	T
10	Tryptophan	Trp	W
	Tyrosine	Tyr	Y
	Valine	Val	V

The following discussion outlines procedures that are useful in identifying proteinoids that are translated from brain-specific messenger RNA (mRNA), and for preparing synthetic polypeptides whose amino acid residue sequences correspond substantially to the amino acid residue sequences of at least a portion of those proteinoids, as well as the manufacture of receptor molecules from those synthetic polypeptides.

#### I. GENERAL APPROACH

The complexity of messenger RNAs expressed in the brain is higher than in other tissues, consequently a large number of tissue specific mRNA molecules may be easily identified. In addition, because the proteins of eukaryotes are colinear with their mRNA, but not necessarily colinear with their genes, brain mRNA, rather than genomic DNA is an appropriate starting part for study.

The adult male rat was chosen herein as a model since it is extensively used for behavioral, neurophysiological, biochemical and neuranatomical studies. In addition, the similarity of brain tissue structure and function among the mammals permits

extrapolation of useful results from the rat to humans and other mammals, as is known.

5       The cytoplasmic poly(A)<sup>+</sup> mRNA from the brains of adult male Sprague-Dawley rats was purified and then converted into complimentary DNA (cDNA). The cDNA, after size enrichment and annealing to the Pst I site of plasmid pBR322, was then used to transform Eschericia coli. Recombinant plasmid DNA was isolated from small overnight cultures of individual cDNA clones, radioactively labeled by nick translation, and was used to probe for complimentary mRNA sequences on Northern blots with samples of brain, liver and kidney poly(A)<sup>+</sup> mRNA.

15       The hybridization patterns of the cDNA clones fell into four classes: those hybridizing equally to all three tissues (Class I; 18%); those hybridizing differently to the three tissues (Class II; 26%); those hybridizing only to brain mRNA (Class III; 30%); and those showing no detectable hybridization (Class IV; 26%).

20       Tables 1-4 (below) list the clones prepared as classified by the tissue distribution patterns of the mRNAs they detected. These data are summarized in Table 5 (below).

25       The abundance of the mRNA molecules corresponding to several of the clones was independently assessed by hybridizing alkali-broken <sup>32</sup>P-end-labeled poly(A)<sup>+</sup> brain mRNA to excess purified plasmid DNA. Those results are also shown in Tables 1-4 hereinbelow.

30

Table 1

Characterization of Class I Clones

<u>Clone</u>	<u>Relative Abundance</u> <sup>1</sup>	<u>mRNA Size</u> <sup>2</sup>	<u>Clone</u>	<u>Relative Abundance</u> <sup>1</sup>	<u>mRNA Size</u> <sup>2</sup>
1B211	3.0	600	1B365	0.05	650
1B447	2.0	1300	1B368	0.05	1000
1B364	1.0	800	1B435	0.05	1200
1B15	1.0	1000	2A361	0.05	1500
1B258	1.0	1300	1B446	0.05 <sup>3</sup>	2000
1B258	1.0	3000	1B446	0.05 <sup>3</sup>	4000
2A559	0.5	600	1B374	0.02	1000
1B359	0.5	1500	1B243a	0.02	2000
1B372	0.5	2400	1B871	0.02	2200
1B203	0.2	400	0-35	0.02 <sup>3</sup>	2500
2A154	0.2	700	1B905	0.02	3000
2A111	0.1	1200	1B7	0.01 <sup>3</sup>	1600
1B268	0.1	1400	2A344	0.01	2500
1B334	0.1	1400	2A361	0.01	2500
1B374	0.1	1500	1B338	0.01	2800
1A124	0.1	2000	0-25	0.01	4000
1A71	0.1	3200			

<sup>1</sup> Estimated relative abundance expressed as a percentage of the clones prepared. Clones that hybridize to a plurality of mRNAs are listed with each mRNA with which they hybridize.

<sup>2</sup> Number average of nucleotide bases.

<sup>3</sup> Abundance determined by filter hybridization.

Table 2  
Characterization of Class II Clones

<u>Clone</u>	<u>Relative Abundance</u> <sup>1</sup>	<u>mRNA Size</u> <sup>2</sup>	<u>Clone</u>	<u>Relative Abundance</u> <sup>1</sup>	<u>mRNA Size</u> <sup>2</sup>
0-31	1.0	1400	2A372	0.1	6000
1B327	1.0	1400	0-29	0.05	1500
1B868	1.0	1400	1B209	0.05	2000
1B330	1.0	2500	1B376	0.05	2000
2A558	0.5	800	2A388	0.05	2500
1B350	0.5	1600	2A261	0.05	2500
1B267	0.5	1700	1B212	0.05	2500
2A290	0.5	1900	0-42	0.05	2500
1B360	0.5	2200	0-42	0.05	3000
0-44	0.2	600	1B347	0.05	3000
2A384	0.2	700	1B227	0.05	3500
1B366	0.2	1700	0-42	0.05	3500
1B328	0.2	2000	0-42	0.05	4000
1B330	0.2	4000	1B347	0.05	4000
2A278	0.1	1200	1B426a	0.05	4000
2A261	0.1	1300	2A345	0.05	5000
1B25	0.1	1300	2A550	0.02	1400
2A346	0.1	1600	2A366	0.02	3500
2A548	0.1	1600	2A383	0.01	1200
0-38	0.1	1700	2A388	0.01	1200
1A188	0.1	2000	1A74	0.01	1700
1B354	0.1	2000	1B867	0.01	2500
0-26	0.1	2500	1B20	0.01	3000
0-38	0.1	3000	1B260	0.01	4000
1A76	0.1	3200			

<sup>1</sup> Estimated relative abundance expressed as a percentage of the clones prepared. Clones that hybridize to a plurality of mRNAs are listed with each mRNA to which they hybridize.

<sup>2</sup> Number average of nucleotide bases.

Table 3

Characterization of Class III Clones

<u>Clone</u>	<u>Relative Abundance</u> <sup>1</sup>	<u>mRNA Size</u> <sup>2</sup>	<u>Clone</u>	<u>Relative Abundance</u> <sup>1</sup>	<u>mRNA Size</u> <sup>2</sup>
0-27	2.0 <sup>3</sup>	160 <sup>4</sup>	1A168	0.05	4000
1B224	2.0 <sup>3</sup>	160 <sup>4</sup>	0-40	0.05	4000
2A120	2.0 <sup>3</sup>	160 <sup>4</sup>	1A273	0.05	4500
1B308	2.0 <sup>3</sup>	160 <sup>4</sup>	1A186	0.05	4500
1B337	2.0 <sup>3</sup>	160 <sup>4</sup>	2A218	0.05	4500
1B208	2.0	3200	1B356	0.05	4500
1B208	1.0	1600	1B346	0.05	5000
1B431	1.0	2000	1B205	0.05	5500
1B373	0.5	2400	1B243b	0.02 <sup>3</sup>	3000
1B426b	0.2 <sup>3</sup>	1400	0-30	0.02	3500
2A278	0.1	1500	2A543	0.02	5000
1B207	0.1	1600	1B236	0.01 <sup>3</sup>	1700
1A75	0.1 <sup>3</sup>	1700	1B261	0.01 <sup>3</sup>	2500
1B213	0.1	2000	1B369	0.01	3200
1B238	0.1	2500	1B435	0.01	3500
1B335	0.1	3200	1B304	0.01	4000
1B424	0.1	3500	2A355	0.01 <sup>3</sup>	4000
1A216	0.05	1700	1B401	0.01	4000
0-29	0.05	2000	2A302	0.01	4000
1A211	0.05	2500	1B353	0.01	5500
2A563	0.05	2500	1B352	0.01	6000
2A355	0.05	2500	1B315	0.01	6000
0-17	0.05	3000	2A347	0.01	6000
1B319	0.05	3000	2A393	0.01	6000
1B361	0.05	3000	2A345	0.01	8000
2A120	0.05	3200	2A345	0.01	10000
1B361	0.05	3300			

<sup>1</sup> Estimated relative abundance expressed as a percentage of the clones prepared. Clones that hybridize to a plurality of mRNAs are listed with each mRNA to which they hybridize.

<sup>2</sup> Number average of nucleotide bases.

<sup>3</sup> Abundance determined by filter hybridizations.

<sup>4</sup> Clones hybridize to the same, repetitive nucleotide sequence (ID sequence) of 160 residues.

Table 4Characterization of Class IV and V Clones

		<u>Class IV</u>	<u>Class V</u>
	Southern positive <sup>1</sup>	19	--
5	Southern negative <sup>2</sup>	--	12
	Untested--36 clones <sup>3</sup>	22 <sup>4</sup>	14 <sup>4</sup>
	Peculiar	---	11
	Total	41	37
<hr/>			
10	<sup>1</sup> Clones exhibiting negative Northern blot analyses and positive Southern blot analyses.		
	<sup>2</sup> Clones exhibiting negative Northern blot analyses and negative Southern blot analyses.		
	<sup>3</sup> Clones not analyzed by Southern blot.		
15	<sup>4</sup> Estimated numbers of clones in each class.		

---

20

25

30

35

Table 5: Summary of Clones by Classes

	Class	Clones <sup>1</sup>	mRNA <sup>2</sup>	Percent of		number
				Total brain mRNA <sup>3</sup>	size avg. <sup>4</sup>	avg. <sup>5</sup>
5	I	29 (18%)	33	11.95	1780	1250
	II	41 (26%)	49	9.55	2350	1870
	III	47 (30%)	48	6.51	3660	2640
			(49) <sup>6</sup>	(8.51) <sup>6</sup>		
	IV	41 (26%)	(41) <sup>7</sup>	--	--	--
10	V	37	--	--	--	--
	TOTAL	191 <sup>8</sup>	130	28.01	2690	1790
			(171) <sup>7</sup>	(30.01) <sup>6</sup>		

<sup>1</sup> Number of clones prepared and percentage of the prepared clones.

<sup>2</sup> Number of mRNAs that hybridize with the prepared clones.

<sup>3</sup> Percentage of total brain mRNA represented by the prepared clones.

<sup>4</sup> Arithmetically computed average mRNA length for each class.

<sup>5</sup> Sum of the product of size and abundance divided by the sum of the abundances.

<sup>6</sup> Value includes the ID sequence.

<sup>7</sup> Value includes presumed, but undetected Class IV mRNAs.

<sup>8</sup> Value includes four clones that fall into more than one class.



Of the four classes of clones, those clones of Classes III and IV are of import herein. Class III clones (43 of 191) hybridized to mRNA species detected in brain but not in liver or kidney, and are exemplified by clone p1A75 (described in detail hereinafter). These clones correspond to 48 mRNA species that are presumed to represent the mRNAs for proteinoids expressed in the neurons and/or glia of the brain, but are not required in liver or kidney. Furthermore, some of these Class III mRNAs produce proteinoid derivatives found only in a subset of brain cells, thereby suggesting that the mRNA is expressed only in that subset of cells.

Additionally, five Class III clones hybridized to a 160 nucleotide base brain-specific, cytoplasmic, poly(A)<sup>+</sup> mRNA target, even though the cDNA insert in each clone is 500-1250 nucleotide bases long. These five clones represent precursors of brain-specific mRNAs which are a small (2-3 percent) part of the original poly(A)<sup>+</sup> cytoplasmic mRNA preparation, but contain a repetitive sequence element (ID sequence) which is also found in the small brain-specific mRNA molecule. [Sutcliffe et al., Proc. Natl. Acad. Sci. USA, 79, 4942-4946 (1982)].

Sixty-seven of the 191 clones failed to detect an mRNA target in the brain, liver or kidney. These clones are thought to represent either plasmids that do not contain cDNA copies of rat brain mRNA and are somehow artifactually present in the clone collection, or they could represent plasmids containing cDNA copies of relatively rare mRNAs.

The presence of rat genetic information in the plasmids was tested for by using nick translated plasmid to probe Southern blots of rat DNA [Southern,

J. Mol. Biol., 98, 503-517 (1975)] to distinguish between those possibilities. Of 31 clones selected at random from the 67 Northern blot negative clones, 19 (61%) hybridized to restriction fragments in Southern blots of rat liver DNA suggesting that those 19 clones did correspond to rare mRNAs transcribed from rat genes.

Because the 31 clones were selected randomly from the 67 Northern blot negative clones, it is presumed that 61%, or 22 clones of the remaining 36 untested clones also correspond to rare mRNAs transcribed from rat genes. Thus, a total of 41 clones correspond to rare brain mRNAs and those 41 clones comprise the Class IV clones.

The remaining 26 Northern blot-negative clones are believed to be artifacts of cloning and have been assigned to a Class V (Table 4). Class V also includes 11 clones that showed peculiar hybridization patterns and could not be classified along with the clones of Classes I-IV. Usually, the peculiar hybridization patterns were smears with brain, liver and kidney mRNAs showing no discrete bands. Presumably, the smeared hybridization pattern is due to hybridization of the poly(T) sequence in the plasmid to the poly(A) tails present in the mRNAs of all size classes and all three tissue mRNA preparations.

In sum, a total of 191 clones have been examined of which 154 correspond to 171 mRNAs (including the 41 presumed Class IV mRNAs). Together, these clones account for more than 28 percent of the total cytoplasmic poly(A)<sup>+</sup> mRNA (by mass) of the rat brain. An additional 2 percent of the poly(A)<sup>+</sup> mRNA is accounted for by the brain-specific 160 nucleotide base mRNA. The

fraction of clones falling into each class is indicated in Table 5, above. The largest fraction is that of the brain-specific Class III clones (30%). Presuming that all or most of the Class IV clones  
5 also correspond to rare brain-specific mRNAs, then more than half of the clones appear to correspond to mRNAs uniquely expressed in the brain.

The arithmetic and number average sizes of the mRNAs listed in Tables 1-3 were computed for each  
10 class and for overall brain mRNAs (Table 5). The arithmetic average represents the average length of the mRNAs assessed by the probes used. The number average reflects the average length of the population of mRNAs represented by the clones; it is weighted by  
15 the abundances of each particular mRNA and is the appropriate figure to compare with measurements of the average length of a heterogenous mixture of mRNA molecules such as those used in solution hybridization determinations of mRNA complexity.

20 The overall number average length of brain mRNAs detected by the clones (Classes I-III) is 1790 nucleotide bases, a figure compatible with the measurements of previous authors, which ranged from 1400-1900 nucleotide bases. [See Bantle et al.,  
25 Cell, 8, 139-150 (1976); Hastie et al., Cell, 9, 761-774 (1976); and Gros et al., In Molecular Genetic Neuroscience, Schmitt, Bird and Bloom, eds., Raven Press, New York, 335-347 (1982).]

The arithmetic average length of the set of  
30 Class III clones is considerably larger, 2640 nucleotide bases. The length of the Class IV clones is presumed to be still larger, averaging about 4960 bases. This indicates that the most prevalent mRNAs in the brain tend to be smaller than the rarer  
35 mRNAs. The same conclusion holds when the two

-27-

averages are computed for mRNAs of each individual class, namely the more prevalent mRNAs tend to be shorter than the rarer mRNAs. Surprisingly, Class III mRNAs are, on average, more than twice as large as those expressed equally in other tissues.

The abundances of the mRNAs corresponding to the clones of Table 1-3 were not measured directly for each clone. Rather, the abundance of the mRNA corresponding to 11 clones was measured directly, and the relative abundances of the other clones were based upon a comparison of hybridization probe-specific activities, autoradiographic exposure times and relative band intensities to the directly measured cases. The resulting, estimated abundances were rounded off so that each mRNA was placed into one of several discrete abundance classes. Hence, the abundance estimates are believed to be accurate to a factor of about two. Furthermore, when the estimates fell below about 0.01 percent, those estimates were rounded upwardly to about 0.01 percent.

Several of the 47 clones of Class III were selected for more detailed study as is described hereinafter. For each, the approach was to determine its nucleotide sequence, followed by the use of purified, single end-labeled restriction fragments from the clone to determine the sense strand of the corresponding mRNA. Potential triplet open reading frames (orfs) were then decoded into amino acid residue sequences using the well-known genetic code. The resulting hypothetical protein sequences were thereafter compared by computer analysis to the collection of known protein sequences to detect possible identities or similarities with previously known proteins. Finally, the open reading frames

which appeared most likely to be translated by the cell into proteinoids were selected, and short polypeptides corresponding to regions from these hypothetical amino acid residue sequences were  
5 chemically synthesized and used as immunogens in rabbits. The resulting antisera were used to detect proteinoids or derivatives thereof in fixed brain sections using immunocytochemical techniques.

## II. CHARACTERIZATION OF EXEMPLARY BRAIN-SPECIFIC CLONES

10 Four brain-specific clones of Class III are described hereinafter as exemplary. These clones are designated plA75, plB236, plB208 and p0-40. The general properties of these clones are summarized in Table 6 hereinafter.

15 The clone insert sizes of 1126, 1500, 978 and 1200 bases respectively were derived from the nucleotide sequence of each insert. The mRNA sizes were estimated from the mobility on agarose gels of the brain-specific mRNA target to which each clone  
20 hybridized in a Northern blot. The mRNA abundance was determined by measuring the amount of broken,  $^{32}\text{P}$ -labeled poly(A)<sup>+</sup> mRNA which hybridized to excess plasmid DNA.

In brief, these four DNA clones represent  
25 mRNA molecules of 1600-4000 nucleotide bases, each of which is present at a concentration ranging roughly from about 0.01 percent to about 2 percent of total brain mRNA. Based upon the sensitivity of the blot utilized herein, it is estimated that the level of  
30 the corresponding mRNA in liver or kidney is less than one copy per cell, assuming about 200,000 mRNA molecules per cell. Thus, the mRNAs of the four clones and those of Classes III and IV are substantially absent from other body tissues such as  
35

liver and kidney, or put differently, the mRNAs are present substantially only in brain cell tissues.

As the brain is comprised of many distinguishable structures and cell types, cDNA clones were used as probes for hybridization to mRNA preparations of the brain from seven sub-regions (cortex, olfactory bulb, hippocampus, hypothalamus, caudate nucleus, cerebellum and pons/hind brain) to define any further regional specificity. The clones were also hybridized to mRNA from tumors of neural origin [a mouse neuroblastoma, rat pheochromocytoma (PC12) and a rat glioma] to determine any cellular specificity. These results are discussed later and are also summarized in Table 6.

Table 6

Properties of Clones plA75, plB236, plB208 and pO-40

	<u>Clone</u>	<u>Insert</u> <sup>1</sup>	<u>mRNA Length</u> <sup>2</sup>	<u>mRNA Abundance</u> <sup>3</sup>	<u>mRNA Location</u> <sup>4</sup>
	PlA75	1126	1700	0.1	all brain, PC12 cells
	plB236	1500	1700	0.01	mid brain, hind brain
	plB236	1500	3200	2	hind, mid and forebrain
	plB208	978	1600	1	glioma cells
	pO-40	1200	4000	0.05	all brain

<sup>1</sup> Length of nucleotide sequence of each clone inserted into the plasmid.

<sup>2</sup> Number average length in nucleotide bases.

<sup>3</sup> Estimated abundance as a percentage of total brain mRNA.

<sup>4</sup> Location in the brain at which the mRNA is present.

III. THE CLONESA. Clone plA75

The plA75 cDNA clone hybridized to a 1700 nucleotide base pair mRNA present at about 0.1 percent of total rat brain cytoplasmic poly(A)<sup>+</sup> mRNA and is not present in either liver or kidney poly(A)<sup>+</sup> mRNAs as can be seen from examination of Figure 1A. The mRNA corresponding to the plA75 clone is expressed about equally in all seven dissected regions of the brain as can be seen from a examination of Figure 1B. The failure to hybridize to liver and kidney mRNA preparations is not due to degradation of mRNAs from those tissues because another cDNA clone (plB15) whose mRNA is not tissue regulated, hybridized as well to the same mRNA preparations of those non-neural tissues as it does to the brain (data not illustrated).

In the regional preparations of brain mRNA, the non-regulated mRNA hybridizing to plB15 acts as an internal normalizer to show that approximately equal amounts of mRNA were loaded in each gel lane. Hence, different levels of hybridization represent real differences in the concentration of the plA75 mRNA in the various tissues. The apparent enrichment of the plA75 mRNA in cerebellum is accompanied by an equal increase in the control plB15 signal, and so must reflect more total mRNA applied to the gel rather than an increase in plA75 mRNA concentration.

The plA75 mRNA is expressed in PC12 cells but not in glioma or neuroblastoma as is seen in Figure 1A. Therefore plA75 is likely to correspond to mRNA that translates to a proteinoid produced in neurons and possibly neuroendocrine cells but not in glia.

Two restriction fragments from each cDNA insert were prepared to determine the coding strand of the 1126 nucleotide base plA75 sequence (Figure 1C). Each fragment was  $^{32}\text{P}$ -labeled on one of the two alternative 5' ends so that a hybridization probe for each of the two possible mRNA orientations was prepared as is shown in Figure 1D.

One probe hybridized to the 1700 nucleotide mRNA on a Northern blot, showing that it was labeled on the strand complimentary to the mRNA; the other probe did not hybridize as is shown in Figure 2E. This result demonstrates that the sequence shown in Figure 1C corresponds to the coding strand of the mRNA. Thus, clone plA75 is a 1126 residue sequence of a portion of the sense strand of a brain-specific 1700 nucleotide base mRNA that represents about 0.1 percent of total brain mRNA.

The nucleotide sequence was then scanned for translation initiator triplets (ATG, GTG) and terminator triplets (TGA, TAA, TAG), as shown in Figure 1D, to determine the nature of the protein could be translated from this mRNA. The well known genetic code was used to decode any sizable possible open reading frames (orfs) into hypothetical proteinoids.

There are only two such large orfs in the plA75 sequence, both of which have been decoded into proteinoid sequences from the sense strand of the cDNA sequence as is shown in Figure 1C. One possible frame (orf 1) is unbounded on the 5' end and extends for 82 triplets until reaching a terminator. The other frame (orf 2) is 119 triplets in length, and is completely contained within the plA75 sequence.

Since it is not known from the sequence itself exactly how the 1126 nucleotide bases of cDNA



-32-

of clone plA75 are aligned within the 1700 nucleotide bases of the mRNA, e.g. 5', middle or 3', orf 1 could potentially be translated by the cell into a proteinoid of up to about 30 kilodaltons (30 K daltons) while orf 2 potentially translates to a proteinoid which must be about 13 K daltons. A computer search of each of the two hypothetical sequences, in overlapping segments of 30 residues revealed no homologies with any known protein sequences. Dayhoff et al., PSQ Version 4, April 30, 1982, National Biomedical Research Foundation, Georgetown University Medical Center (hereinafter referred to as the "Dayhoff atlas").

Two approaches were used to determine if either of the hypothetical orf sequences corresponds to a brain-specific proteinoid. Denatured plA75 DNA was bound to nitrocellulose filters. The filters so prepared were then used to purify complimentary mRNA by hybridization with total brain poly(A)<sup>+</sup> mRNA. After extensive washing of the filter, the bound mRNA was released by boiling and used to program in vitro protein synthesis (hybrid-release translation) as described by Cleveland et al. Cell, 20, 95-105 (1980).

The purified mRNA stimulated the synthesis of a proteinoid with a gel mobility of approximately 25 K daltons which was not present in control reactions (Figure 2A). Unpurified brain poly(A)<sup>+</sup> mRNA programmed into synthesis of many proteinoids. From these determinations, the size of this molecule (25 K daltons) suggested that orf 1 is likely to be translated into the product of the plA75 mRNA because orf 2 and any other possibility was too small.

Two 13-residue synthetic polypeptides, P1 and P2 corresponding to two amino acid residue

-33-

sequences within orf 1 were chemically synthesized to detect the putative product of the pIA75 mRNA translation. The polypeptides underlined in Figure 1C, were chosen so as to contain proline residues and charged residues, using the rules discussed by Sutcliffe et al., Science, 219, 660-666 (1983). Those polypeptides were then coupled to a variety of carriers as discussed in the Materials and Methods Section. A synthetic polypeptide, denominated P3, was also synthesized from orf 2. Synthetic polypeptides P1, P2 and P3 are hereinafter sometimes referred to as P1, P2 and P3', without reference to their being synthetic polypeptides.

The amino acid residue sequences of the three polypeptides were compared to the Dayhoff atlas, above, by computer database search to preclude the possibility that at reasonable concentrations, antisera to any of these three polypeptides would specifically interact with any protein of a known sequence. None of the three polypeptides were found to have a close homolog.

Antisera to the polypeptide-carrier conjugates were raised in rabbits. The sera were shown to react strongly with the appropriate polypeptides by ELISA following the procedures of Green et al., Cell, 28, 477-487 (1982).

The antisera were then used to probe extracts of <sup>35</sup>S-methionine labeled PC12 cells which produce pIA75 mRNA. Thereafter, antisera to synthetic polypeptide P1 was found to react with a proteinoid with a slightly diffuse gel mobility of about 28 K daltons (Figure 2B). Anti-synthetic polypeptide P2 serum showed a lower reactivity against a proteinoid with the same gel mobility, but anti-synthetic polypeptide P3 sera were unreactive (data not shown).

Orf 1, therefore, is the proteinoid coding frame of the mRNA corresponding to plA75. Since only 82 triplets of orf 1 are at the 5' end the cDNA sequence, and orf 1 is translated to part of 28 K dalton product, plA75 must represent a 3' region of a corresponding mature mRNA (Figure 2C). There is, therefore, a long 3' untranslated region on this mRNA exceeding about 800 nucleotides. Approximately 450 nucleotides of the 5' prime coding sequence are therefore lacking.

To determine in which cells in the brain the plA75 orf proteinoid is expressed, thin, fixed sections of rat brain were reacted with anti-P1, anti-P2 and anti-P3 sera. The sections were washed, and antibody-antigen (receptor-ligand) complexes were visualized with horseradish peroxidase (HRP)-coupled anti-rabbit immunoglobulin.

One antiserum against synthetic polypeptide P1 reacted with the cell bodies of some neurons located throughout the brain (Figures 3A-3C), including many large neurons such as cortical pyramidal cells. The reactivities appear to be cytoplasmic and have a granular appearance. Frequently, the granules are concentrated in the dendritic pole of the cell and are often observed in the dendrites themselves (Figure 3C). The reacting cell bodies were found in the cerebellum, deep cerebellar nuclei, hypothalamus, superior colliculus, subiculum and cerebral cortex.

Anti-P2 sera gave a similar pattern of cytoplasmic staining, but it was limited to a small group of cells in the hypothalamus (Figure 3D). Those data, together with the anti-P1 staining pattern, are interpreted to mean that the proteinoid in the hypothalamus is somehow more immunoreactive,

and that the anti-P2 sera are weaker as noted for the immunoprecipitation of PC12 extracts.

The anti-P2 immunoreactivity was blocked by preincubation of the antiserum with synthetic polypeptide P2. The reactivity of the anti-P1 serum could not be blocked with synthetic polypeptide P1, with the P1-thyroglobulin conjugate to which the antiserum was raised or with thyroglobulin alone.

Since the distinct cytoplasmic granular nature of the staining is the same throughout the brain and looks similar with the antisera to the two polypeptides in the hypothalamus, and since both antisera react with proteinoids of the same size in extracts of PC12 cells, both anti-P1 and anti-P2 sera are probably reacting with the same proteinoid or derivative thereof in the brain; i.e., the 28 K dalton product of the plA75 mRNA seen in the PC12 extracts.

Antibodies against synthetic polypeptide P3 were not reactive in any brain region. That result is consistent with the conclusion that orf 2 is not the true coding region of plA75.

The distribution of anti-P1 and anti-P2 immunoreactivity is in general accord with the regional localization of plA75 mRNA as shown in Figure 1B, the reactive cell bodies being located throughout the brain. The staining was not observed in all cells, but was limited to a subset of neurons which appear to be morphologically related: many of the reactive cells are large projection neurons such as pyramidal cells. The cytoplasmic location of the reactive proteinoids within the cells, and the granular appearance and polar orientation of the immune reaction, suggest that this proteinoid or derivative thereof could be involved in the synthesis

or directional export of proteinaceous or materials destined for dendrites, or it could be a component of cytoplasmic organelles, such as mitochondria. These results therefore document the discovery of a 28K  
5 dalton proteinoid that is expressed selectively in roughly homologous neurons and whose carboxy-terminal 82 amino acid residues are now known.

The antibodies to synthetic polypeptides P1 and P2 or idiotype containing polyamides made  
10 therefrom are thus useful receptors for the study of the synthesis of the 28 K dalton protein in PC12 or other nerve cells, and are useful to define the location of that proteinoid or its derivative in brain cell tissue by more detailed  
15 immunocytochemistry and electron microscope studies. These specific receptors (antibodies or idiotype-containing polyamides made therefrom) can also facilitate the purification of the endogenous brain cell tissue products by conventional  
20 biochemical means for further sequence determination and assay of potential function. The organelle-like location of the proteinoid within cells suggests an involvement in secretory, transport or mitochondrial functions, and gives important clues as to how the  
25 determination of the function of this proteinoid may be approached.

The amino acid residue sequence of synthetic polypeptides P1, P2 and P3 are as represented by the formulas below, from left to right and in the  
30 direction from amino-terminus to carboxy-terminus, using conventional single letter code for amino acid residues:

P1 CIPEGLESYYTEQ;  
P2 RSVSPWMSVLSEE; and  
35 P3 CRYPPIHRVTPRS.—

-37-

B. Clone plB236

The plB236 cDNA clone hybridizes to a brain-specific 1700 nucleotide mRNA representing 0.01 percent of total brain mRNA (Figure 4A). This mRNA is distributed in low amounts in cortex and olfactory regions, and in larger amounts in mid and hind brain regions (Figure 4B). This mRNA is not expressed in any of the three tumors thus far tested, nor is it expressed in gut, lung, heart or skeletal muscle.

The approximately 1500 nucleotide sequence of the strand depicted in Figure 4C was shown to be the sense of strand of the mRNA by a determination analogous to that described above for clone plA75, utilizing single <sup>32</sup>P-end-labeled restriction fragments of opposite orientations to probe a Northern blot as is shown in Figures 4D and 4E. In addition, since the plB236 sense strand sequence terminates with a poly(A) stretch of over 100 bases, the exact number being uncountable on the sequence gels, and contains a variant poly(A) addition sequence (AACAAA) 17 nucleotide bases upstream, it appears quite certain that this is the proteinoid coding strand.

After scanning the sequence for terminator triplets (Figure 4D), it is clear that the long orf bounded at the 5' end of the clone and extending for almost 1000 nucleotides must be the coding frame. This leaves 450 nucleotides of the 3' non-coding information.

The putative amino acid sequence of 318 residues (indicated above the nucleotide sequence of Figure 4C) although unbounded at its amino-terminus, cannot be too much larger, for the full length of the mRNA is estimated to be only about 200 nucleotides larger than the plB236 cDNA insert. The 318 residue

proteinoid sequence was compared by computer analysis in overlapping sequences of 30 residues each to the proteins of the Dayhoff atlas, above, but no notable homologies were detected.

5           The plB236 DNA was bound to nitrocellulose filters, and complimentary mRNA was purified and translated in vitro as described for clone plA75. However, no specific translation product was observed. This was not altogether surprising since  
10 plB236 hybridizes to a rarer mRNA than the mRNA corresponding to plA75.

          The 318 amino acid sequence of the probable plB236 coding frame is similar to the that of known precursor of pharmacologically active neuropeptides  
15 and is shown schematically in Figure 5. The carboxy-terminal region contains a series of tandem basic amino acids: arg-arg, lys-arg, and arg-arg-lys-lys. Such pairs of basic amino acid residues are proteolytic cleavage sites in the  
20 precursors for many neuropeptides or hormones such as proopiomelanocortin [Nakanishi et al., Nature, 278, 423-427 (1979)] calcitonin [Amara et al., Nature, 298, 240-244 (1982)] and the precursors for  
25 vassopressin/neurophysin [Land et al., Nature 298, 299-303 (1982)] and beta-neoendorphin/dynorphin [Kakidani et al., Nature, 298, 245-249 (1982)]. Furthermore, in most of these examples, the active polypeptide sequences are located in the carboxy-terminal end of the precursor, very similar  
30 to that which is seen herein.

          Three (P5, P6 and P7) of five polypeptides from the regions demarcated by the pairs of basic residues, shown as bars in Figure 5 and underlined in Figure 4C, were prepared synthetically since it was  
35 desirous to identify the pLB236 proteinoid in rat

brain by use of antisera raised to the synthetic polypeptides. Those polypeptides used as immunogens for the preparation of antisera were themselves also studied to ascertain the pharmacological activities.

5 Synthetic polypeptides denominated P4-P8 are also sometimes referred to herein without the descriptive phrase "synthetic polypeptide."

10 Synthetic polypeptide P6 is particularly noteworthy as its carboxy-terminal residue is glycine. Several carboxy-terminally amidated polypeptides such as vasopressin (Land et al., above), calcitonin (Amara et al., above) and alpha-MSH (Nakanishi et al., above), are cleaved from precursors at GlyArgArg sequences, and transamidated,  
15 the glycine being eliminated to leave the newly formed carboxy-terminal residue in the amide rather than carboxy form. Synthetic polypeptide P6 is therefore a candidate for such a post translational modification, and as such provides further structural  
20 indication that its sequence may represent a neuropeptide precursor.

Synthetic polypeptides translated from mRNAs present substantially only in brain cell tissues that translate to a proteinoid or proteinoid derivative  
25 containing a GlyArgArg sequence that may be cellularly transformed by the above cleavage-transamination reaction to provide a carboxy-terminal amide group on the residue to the amino-terminal side of the GlyArgArg sequence; i.e.,  
30 adjacent to the Gly residue, are also particularly contemplated herein. Such synthetic polypeptides consequently do not contain a carboxy-terminal glycine as do P6 and P8. Rather, the carboxy-terminal glycine residue is deleted, and the  
35 carboxy-terminal amino acid residue contains an amide



group, denominated herein as an amino acid residue one-letter symbol followed by "-NH<sub>2</sub>". Such synthetic polypeptides are exemplified by synthetic polypeptides P9 and P10 whose amino acid residue sequences are shown hereinafter.

As in the study of clone plA75, the synthetic polypeptides were coupled to carriers to form conjugates, and the conjugates were used to immunize rabbits. The resulting synthetic polypeptide-reactive antisera were used for immunocytochemical studies. The details of this work are as reported in the Materials and Methods Section hereinafter.

Indirect immunoperoxidase staining, as discussed above, was performed with antisera raised against synthetic polypeptides P4, P5, P6 and P7. Staining with antisera raised against synthetic polypeptide P4 was consistently negative. However, four sera raised against P5 and two sera raised against P6 and one serum raised against P7 have given similar overall patterns of immunoreactivity in rat brain.

Immunoreactivity was found in a fiber system distributed throughout the brain, with distinctive patterns most extensively revealed in pons, cerebellum (Figure 6A), hypothalamus and regions of a hippocampus and neocortex. In cerebellum, hippocampus and neocortex, thick variocose fibers are seen in direct relationship to the perikarya (cell bodies) of the principal output cells of each region.

In the hippocampus and cerebellum, the axon-like processes stained by antisera to P5 thickened, surrounding (and probably making synapses on) the cell body and proximal dendrites of the CA<sub>3</sub>

-41-

pyramidal neurons (Figure 6C) and Purkinje neurons, respectively. Nerve terminal-like process are also found in the cerebellum within the the granule cell layer (Figures 6A and 6B) and surrounding (and  
5 probably making synapses on) a sizable fraction of the large neurons of the deep cerebellar nuclei (Figure 6G). Within the neocortex, immunoreactivity is most intense in the posterior cingulate cortex, with radially-directed axon-like staining in layers  
10 3, 4, 5 and with tangentially directed fibers resembling a terminal field in layer 1 (Figure 6E). A similar pattern is seen in certain regions of the somatosensory cortex where staining is in radially directed fibers spanning layers 2-5, and again  
15 tangentially directed in the outermost zone of layer one.

With antisera to P6, staining in neocortex is similar in all respects (Figure 6F), except that the staining is fainter, and is virtually absent in  
20 the tangentially arranged structures of layer 1. Staining with antiserum to P7 is indistinguishable from staining with antiserum to P5. Additionally, fiber staining is also observed within axons of the heavily myelinated tracts of the fornix, the corpus  
25 striatum, and the lateral olfactory tract, as well as in the hypothalamic tract and the striamedullaris. However, with the exception of the some very faintly reactive cell bodies in the ventromedial and basal arcuate regions of the hypothalamus, immunoreactive  
30 cell bodies are difficult to visualize in fixed brains of untreated rats.

To improve detection of immunoreactivity within cell bodies, rats were treated with colchicine, which blocks axonal transport from  
35 neuronal cell bodies to terminals, causing

transported material to accumulate in the cell bodies. This treatment facilitates immunodetection of the material at its site of synthesis.

In colchicine treated rats, fiber-like staining with antisera to P5 was considerably reduced; this reduction was most obvious within the granule and molecular layers of the cerebellar cortex and within layer 1 of the cingulate and neocortex. Although fiber staining was reduced, distinctive immunoreactive cell bodies were thus observed in which cytoplasm, but not the nucleus became immunoreactive. Such immunoreactive cell bodies (Figure 6H) were clearly discerned in the brain stem (in the medial nucleus of the trapezoid body, the ventro pontine tagmental area, and the nuclei of the pontine raphe system) and more clearly than in untreated rats within the lateral ventro-medial and basoarcuate nuclei of the hypothalamus and the caudate nucleus. The distribution of these immunoreactive cell bodies is consistent with the regional localization of plB236 mRNA.

All immunoreactivity with the antiserum which was used for the majority of the studies on normal and colchicine-treated rats was identical at dilutions of from 1:500 to 1:10000. When the antiserum at a 1:1000 dilution was preabsorbed with synthetic polypeptide P5, all immunoreactivity was abolished (Figure 6D). Similar absorptions of this antiserum with synthetic polypeptides P4 and P6 had no effect on the staining pattern or intensity. Similarly, the anti-synthetic polypeptide P6 reactivity was specifically blocked with synthetic polypeptide P6, and the anti-P7 reactivity was blocked by preincubation with synthetic polypeptide P7. Thus, based on the coincident pattern of

immunoreactivity of antisera to the three synthetic polypeptides, the staining was specific for the plB236 mRNA product.

The selective and specific staining patterns observed with several antisera to synthetic polypeptides P5, P6 or P7 of the plB236 orf deduced from its sequence strongly suggest that an extensive system of neurons with a reproducible pattern of circuitry relationships may contain this proteinoid.

The appearance of immunoreactive cell bodies in colchicine-treated rats, together with a simultaneous loss of fiber staining, further supports the view that the immunoreactive material is synthesized in certain neuronal cell bodies and transported nerve terminals.

Extracts from rat brain cell tissue homogenates were analyzed by Western blot as generally described in Towbin et al., Proc. Natl. Acad. Sci. USA, 76, 4350-4354 (1979). Sera raised to P5, P6 and P7 showed coincident, peptide-blockable reactivity for each serum.

The reactive proteinoid had a diffuse gel mobility of 100K daltons. This proteinoid is glycosylated inasmuch as its apparent size is reduced to 70K daltons after incubation of the brain extract with endoglycosylase F which removes N-linked carbohydrate side chains as discussed by Elder et al., Proc. Natl. Acad. Sci. USA, 4540-4544 (1982). Thus, the proteinoid translated from the mRNA corresponding to clone plB236 is cellularly derivatized by a glycosylation.

Cell fractionation determinations showed that the immunoreactive 100K dalton proteinoid copurifies with a synaptosomal fraction. Dissection studies showed that this proteinoid is located in the

same brain regions as are described in the immunocytochemistry results.

Solublized portions of brain cell tissue extracts were analyzed for the presence of proteinoid derivatives cleaved from the larger proteinoid by the  
5 cell. High pressure liquid chromatography (HPLC) and radioimmune assay techniques using antibody receptors raised to the synthetic polypeptides of this invention were used here. Immune-reacting  
10 proteinoids in the size range of synthetic polypeptide P5 were detected when anti-P5 serum was used in the assay with the HPLC eluate. This result indicates that the plB236 proteinoid is a precursor for smaller proteinoid derivatives.

15 Together with the structure of the plB236 proteinoid, the morphological data strongly suggest that this proteinoid is a precursor for a new neuropeptide. The requirements that a polypeptide must meet to be considered a bona-fide  
20 neurotransmitter are rather strict, and the evidence that segments of the plB236 proteinoid have a neurotransmitter function is circumstantial and preliminary from the above data. Nevertheless, it is believed that the plB236 proteinoid or one or more of  
25 its cellularly processed derivatives are neurotransmitters.

In preliminary studies in which synthetic polypeptides P5 or P8 have been applied iontophoretically in cerebellar Purkinje cells or  
30 hippocampal pyramidal cells, the firing rate of those cells where shown to be modified. The procedure followed for these studies was analogous to that described by French et al., Regulatory Peptides, 1, 127-146 (1980).

More specifically, when P5 at a 1 millimolar concentration was applied by pressure to cortical neurons, there was a decrease in the rate of spontaneous activity as measured by single unit action potentials. Similar application of P5 to cells in the CA<sub>3</sub> region of hippocampus resulted in a marked and prolonged increase in the rate of cell firing. Neither effect was noted when other synthetic polypeptides such as P4 were applied similarly. The electrophysical effects of P5 are therefore specific both for the polypeptide and the anatomical localization of the reactive cells.

Preliminary studies with P5 administered intraventricularly into rat brains in an amount of about 5 micrograms per mouse resulted in a significant increase in locomotor activity that was not found with control polypeptides or with saline. The method used for intraventricular administration was analogous to that described by Koob et al., Regulatory Peptides, 2, 153-163 (1981).

Synthetic polypeptide P8 which contains the amino acid residue sequence of P6 plus an additional six residues at the amino-terminus of P6 exhibited no electrophysiological effect when administered alone iontophoretically. However, when administered at a 1 millimolar concentration in conjunction with the known excitatory transmitter glutamate (0.5 molar), there was a prolongation of glutamate-induced stimulation. This effect suggests that the natural proteinoid or more likely its derivative corresponding to P8 or P6 provides neuroactivity modulatory action.

The antibodies against the p1B236 proteinoids provide useful markers for a major afferent pathway, prominent in cortical regions,

regardless of whether the synthetic polypeptides are themselves physiologically active. As is discussed in more detail hereinafter, neurotransmitter-type activity for the synthetic polypeptides derived from the proteinoid encoded by plB236 has now been shown, and thus this work demonstrates not only a newly discovered major, new neuronal system, but also its putative neurotransmitter.

The amino acid residue sequences of synthetic polypeptides P4, P5, P6, P7, P8, P9 and P10 are represented by the formulas below, from left to right in the direction from amino-terminus to carboxy-terminus, again using the single letter amino acid residue code:

15           P4    LRGQAGAPPRVIC;  
             P5    NVTESPSFSAGDNPHVLYSPEFRISGAPDKYESE;  
             P6    LLGLRGEPPPELDLSYSHSDLG;  
             P7    PTKDSYTLTEELA EYAEIRVK;  
             P8    LGSERRLLGLRGEPPPELDLSYSHSDLG;  
20           P9    LLGLRGEPPPELDLSYSHSDL-NH<sub>2</sub>; and  
             P10  LGSERRLLGLRGEPPPELDLSYSHSDL-NH<sub>2</sub>.  
             C.   Clone plB208

The plB208 cDNA clone hybridizes to two abundant brain-specific mRNAs that are about 3200 and about 1600 nucleotide bases in length (Figure 7A). Both mRNAs appear to be coordinately controlled since the relative intensities of the two bands remain approximately constant as the the concentration of each increases in a gradient from forebrain to hindbrain (Figure 7B). Since the two mRNA species are detected in glioma mRNA but not in PC12 or neuroblastoma mRNA, this is believed to be a clone of glial-specific mRNA. From the intensity of the hybridizations, it is estimated that these two mRNAs species are the two most abundant rat brain-specific

mRNAs thus far detected. The difference in the relative hybridization strengths could either reflect that this clone has greater homology to the larger species, or that the two target species are present in different concentrations.

Both mRNAs have a common sense strand (the 978 nucleotide base cDNA insert sequence is shown in Figure 7C) as indicated by a determination analogous to that for plA75, and detailed in Figure 7D and Figure 7E. Probes complimentary to the 5' end of the sequence (S1V1) hybridized to both target mRNAs. Probes complimentary to the 3' end (S2F2) hybridized only to the longer species, thereby indicating that the region of homology between the two mRNAs is in the 5' portion of the sequence. This also indicates that this clone is a copy of a larger mRNA species.

The only sizable orf is unbounded at the 5' end, and runs into the sequence for 121 triplets (Figure 7D). A putative polypeptide sequence is shown above the nucleotide sequence in Figure 7C. This putitive sequence is not homologous to any proteinoid sequence in the Dayhoff atlas database, above.

As can be seen from the putative sequence, the translated polypeptide is very hydrophobic. Antibodies to this proteinoid have not been pursued.

This hydrophobic polypeptide corresponds to the largest orf in a partial cDNA (978 nucleotides clone of a considerably larger (3200 nucleotides) very abundant glial mRNA. It is believed, from the probable abundance and the hydrophobicity of the putative amino acid residue sequence that the proteinoid encoded from plB208 found in brain cell tissues is glial fibrillary acidic protein (GAFP), a known, prevalent very insoluble 55K dalton glial



structural protein about which little is known at the molecular level.

D. Clone p0-40

The p0-40 cDNA clone hybridizes to a  
5 brain-specific 4000 nucleotide mRNA representing  
about 0.05 percent of brain mRNA (Figure 8A). This  
mRNA has a regional distribution pattern in the brain  
similar to plA575, except for its relatively lower  
concentration in cortex where dedgredation of the  
10 mRNA could possibly have occured (Figure 1B).

The sequence of the p0-40 clone has been  
determined as it corresponds to a part of a large,  
brain-specific mRNA (Figure 8B). The strand  
corresponding to the mRNA coding strand was  
15 identified by determinations analogous to those used  
for the plA75 clone. Neither the coding nor the  
non-coding strand of this clone exhibits a long open  
reading frame (Figure 8C).

It is possible to derive short open reading  
20 frames of similar length to those determined for  
plA75. However, about 3000 nucleotides are lacking  
from the mRNA sequence and it is not known how this  
clone is aligned to bona fide mRNA. As  
brain-specific mRNAs may have larger than normal  
25 non-coding regions, it is believed that this clone  
may have missed the proteinoid coding region of the  
corresponding mRNA. This clone was consequently an  
unlikely choice for further investigation of  
hypothetical proteinoid products.

30 E. General Conclusions

Four cDNA clones corresponding to rat  
brain-specific mRNAs have been studied in detail.  
The relative regional concentrations of the mRNAs and  
nucleotide sequences and protein coding strands for  
35 each clone have also been determined.

For two clones, the proteinoid products of the mRNA have been identified by chemically synthesizing short polypeptides corresponding substantially to regions predicted from the open reading frames on the sense strand of the mRNA, and by raising antisera to the synthetic polypeptides. The antisera have identified, by immunocytochemistry, the particular subsets of cells within the rat brain which contain the protein product of the two brain-specific mRNAs, thus indicating that this approach provides a powerful means for describing and locating brain proteinoids and their derivatives. The antipeptide receptors are useful for further studies aimed at localizing those proteinoids or derivatives within the specific cells and for purifying the proteinoid molecules by conventional biochemical and immunoaffinity approaches.

The above-described approach has some features that are in common with strategies for using a monoclonal antibodies to identify specific brain antigens as described by Barnstable, Nature, 286, 231-235 (1982). However, the present approach offers several advantages.

The antisera generated react with predetermined sites (epitopes) within proteinoids or with proteinoid derivatives whose amino acid residue sequences were determined previously, in part by decoding the clonal DNA. In addition, a nucleic acid probe is provided that is already in hand at the time the proteinoid is sought. Thus, if questions about the gene locus, developmental regulation or other details of gene expression arise, the present system is ready for immediate extension.

Furthermore, by demonstrating coincident reactivities of antisera to two synthetic

-50-

polypeptides from distinct regions of a naturally occurring proteinoid sequence, it is possible to increase the confidence that immunocytochemical reactions occurring at two loci in the brain are a result of the presence of the same proteinoid at those sites. Such is not the case with monoclonal antibodies against brain antigens. Finally, the present approach uses sequence determination as a starting point and as more structural information emerges or becomes interpreted, it is likely that great advances will be made in using sequence to predict function.

Brain-specific mRNAs represent a large portion of brain mRNA content, possibly corresponding to about 30,000 mRNA species, some of which are expressed at a level of about 0.001 percent. For such mRNAs reasonably to play significant biological roles, each must probably be concentrated in a few cell types rather than spread throughout the brain. The proteinoid translation product of the plB236 mRNA is present in a small fraction of brain cells, although its mRNA is estimated to be about 0.01 percent of the total brain mRNA. The immunocytochemical technique appears to have sufficient sensitivity to detect the products of mRNAs expressed at 0.001 percent, given the ease of detection of a proteinoid whose mRNA is tenfold more abundant. Thus, given that Class IV clones correspond to the very rare mRNAs in total brain mRNA that are thought to be localized in small subregions of the brain, the proteinoid products of that most interesting class of brain mRNAs may be detectable using the methods described herein.

The proteinoid products of both plA75 and plB236 are present in subsets of brain cells. That

different specific genes are turned on in different cell subjects of the brain indicates at the molecular level what was already known at the anatomical level; i.e., the brain is a organ made up of many cell types and neurons are a heterogenous cell class whose members have differentiated from common precursors.

The generally low abundance of brain-specific mRNAs (Class III) relative to mRNAs that are not specifically expressed in brain (Classes I and II), and the potentially large number of very low abundance mRNAs (Class IV) suggest that many brain-specific mRNAs are expressed uniquely in subsets of brain cells that have differentiated from their neuronal stem cells. Therefore, brain-specificity per se is a rather primitive classification.

The present studies, based upon antiserum reactivities, provides a more precise, molecularly-based classification system for cell types. Indeed, this work has already identified two novel brain-specific proteinoids and/or proteinoid derivatives. One proteinoid or its derivative forms an immune reaction with anti-P1 and anti-P2 sera, and is located in the cytoplasm of a large portion of brain cells. It may be involved in the protein synthetic or dendritic transport events exclusive of certain large neurons. The second proteinoid or derivative forms an immune reaction with antisera to P5, P6 and P7, and is present in a discrete pathway of fibers leading from the hindbrain to several distinct anatomical sites. That proteinoid itself, or its brain cell-processed derivatives, has neuroactivity and may be a neurotransmitter.

IV. MATERIALS AND METHODSA. Complimentary Deoxyribonucleic Acid  
(cDNA) Cloning and Messenger  
Ribonucleic Acid (mRNA) Procedures

5           Details of mRNA preparation, cDNA cloning  
and the selection of brain specific clones are stated  
below in Sections E and F. For the regional analysis  
of brain mRNA, rat brains were dissected on ice and  
extracted as described for total brain mRNA, below.  
10   mRNA preparations were separated by electrophoresis  
on 1.5 percent agarose gels, in 1 molar formaldehyde,  
and transferred to nitrocellulose as described in  
Thomas, P.S., Proc. Natl. Acad. Sci. USA, 77,  
5201-5205 (1980). The mRNA preparations were  
15   hybridized ("Northern blot") as described in Section  
G with <sup>32</sup>P-labeled plasmid probes prepared by nick  
translation as described in Rigby et al., J. Mol.  
Biol., 133, 237-251 (1977). In some determinations,  
hybridization probes were end-labeled with <sup>32</sup>P  
20   using polynucleotide kinase as described below in  
Section B.

          The procedure described in Cleveland et al.,  
Cell, 20, 95-105 (1980) was used for the positive  
hybridization-translation assay (HART): mRNA was  
25   translated in vitro in a rabbit reticulocyte lysate  
system (New England Nuclear, Boston,  
Massachusetts). Products were analyzed by sodium  
dodecyl sulfate (SDS) gel electrophoresis as  
described in Laemmli, U.K., Nature, 227, 680-682  
30   (1971).

          The rat C6 glioma and mouse C1300  
neuroblastoma cell lines were obtained from the from  
the American type culture collection; rat  
pheochromocytoma (PC12) cells were obtained from  
35   Dr. Joel Levine of the Salk Institute.

-53-

B. Nucleic Acid Sequencing

Using the procedure described in Tanka et al., J. Bacteriol., 121, 354-362 (1975), plasmid DNA was prepared from chloramphenicol-treated cultures of bacteria carrying the cDNA clones which were identified by the procedure described below in Section F. Aliquots (1 microgram) of each plasmid were digested with each of nine restriction endonucleases having tetra- or penta-nucleotide recognition sequences (Alu I, Bst IV, Hae III, Sau 3a, Hpa II, Hinf I, Ava II, Rsa I, FnuD II). The electrophoresis patterns of the digestion products were compared with those of the plasmid pBR322 as described in Sutcliffe, J.G., Nucleic Acid Res., 5, 2721-2728 (1978), thereby allowing the identification and sizing of restriction fragments containing the cDNA insert.

Preparative restriction digests (50 microgram) were made with two enzymes that gave 3 or 4 insert-containing fragments. Those fragments were treated with phosphatase, purified on polyacrylamide gels, and were end-labeled with  $^{32}\text{P}$  using polynucleotide kinase. Small aliquots (1 percent) of the end-labeled fragments were digested with several of the enzymes initially screened, and products were displayed on polyacrylamide gels.

From the sizes of the product fragments detected by autoradiography it was generally possible to construct a reasonably accurate restriction map of the insert. This map was used to determine how to cleave the remaining radioactive fragments prior to sequence analysis as described in Maxam et al., Proc. Natl. Acad. Sci. USA, 74, 560-564 (1977) such that the resulting data were likely to saturate the insert and, if possible, provide overlaps and duplicate

-54-

sequence determinations for all regions. Usually, this strategy was sufficient to provide enough sequence data such that only a few specific fragments needed to be prepared for completing a verified  
5 insert sequence. This technique allowed several cDNA inserts of 1000-1500 base pairs (bp) to be sequenced in one month. Some of the single end-labeled products of this procedure were used in Northern blots to determine the strandedness of the  
10 corresponding mRNAs.

### C. Antipeptide Sera

Regions of the cDNA clone p1A75 open reading frame (orf) 1 and orf 2, and the cDNA clone p1B236 orf were scanned for charged regions in the vicinity  
15 of proline residues as described in Sutcliffe et al., Science, 219, 660-666 (1983). Corresponding synthetic polypeptides P1, P2, P3 and P4 were prepared as described in Bittle et al., Nature, 298, 30-33 (1982). Synthetic polypeptides P5, P6, P7 and  
20 P8 were prepared as were polypeptides P1-P4, and were selected because of their potential roles as neuroactive materials (Figure 5). All polypeptides were used without further purification as their initial use was in obtaining specific antisera.

25 Each polypeptide was coupled to three carriers, keyhole limpet hemocyanin (KLH), edestin and thyroglobulin by gluteraldehyde treatment: 5 milligrams polypeptide and 5 milligrams carrier protein were suspended in 0.7 milliliters of 0.25  
30 molar sodium phosphate buffer (pH 7.2), incubated with 4 microliters 25 percent gluteraldehyde for 30 minutes at room temperature, and the resulting polypeptide-carrier conjugate was purified by chromatography on Sephadex G-50 (a beaded,  
35 cross-linked carbohydrate polymer, Pharmacia Fine Chemicals, Piscataway, New Jersey).

The resulting conjugates were used to immunize rabbits. Sera were assayed for antipeptide activity by enzyme-linked immunosorbent assay (ELISA) as described in Green et al., Cell, 28, 477-487 (1982).

#### D. Immunocytochemistry

The procedures for tissue preparation and immunocytochemistry were essentially as described for the detection of beta-endorphin and gamma-melanocyte-stimulating hormone in Bloom et al., Proc. Natl. Acad. Sci. USA, 75, 1591-1595 (1978). Rats were anesthetized and perfused transcardially with chilled, freshly depolymerized 5 percent formaldehyde in phosphate buffer (0.3 molar, pH 7.4). After dissection into coronal and sagittal slabs, the tissue was immersed in fixative for 3 hours and passed through increasing concentrations of sucrose (12 to 18 percent) in phosphate buffered saline (Pi/NaCl; 0.15 molar phosphate buffer, pH 7.4, 0.15 molar NaCl).

Sixty-micron thick sections were cut in a cryostat and collected in Pi/NaCl. These sections were incubated free floating with appropriate dilutions of the primary antibody in Pi/NaCl containing 1 milligram per milliliter bovine serum albumin and 0.3 percent Triton X-100 (polyoxyethylene-9 octyl phenyl ether), Rohm & Haas Co. (Philadelphia, Pennsylvania) for 12 to 18 hours at 4 degrees C. with continuous vibration. After washing in Pi/NaCl, the sections were incubated for 2 hours at room temperature with vibration along with 2 micrograms per milliliter goat anti-rabbit IgG conjugated to horseradish peroxidase.

After a further washing in Pi/NaCl, the sections were developed with 0.5 percent



-56-

diaminobenzidine, 0.003 percent  $H_2O_2$  for 8 to 15 minutes at room temperature, washed, air dried. They were then examined under a microscope. Some sections were counterstained with cresyl violet or with Richardson's stain to visualize cell nuclei. For preabsorption of the antibodies with polypeptides, dilutions of antibodies were incubated with 60 micrograms per milliliter polypeptide overnight at 4 degrees C. and then applied to tissue sections as described above.

Rats were treated with colchicine to enhance the detection of immunoreactivity in neuronal cell bodies (50 colchicine micrograms per rat in 50 microliters of Krebs-bicarbonate solution 127 millimolar NaCl, 3.83 millimolar KCl, 1.8 millimolar  $CaCl_2$ , 1.18 millimolar  $KH_2PO_4$ , 1.18 millimolar  $Mg SO_4$ , 20 millimolar  $NaHCO_3$ , 2 grams per liter of d-glucose, 0.1 percent bovine serum albumin, gassed with a mixture of oxygen and carbon dioxide at an  $O_2:CO_2$  ratio of 95:5, pH value of 7.4)) by stereotaxic intracisternal injection under chloral hydrate anesthesia. The treated rats were perfused and prepared for immunocytochemistry 48 to 72 hours later by an otherwise unchanged procedure from that above.

When keyhole limpet hemocyanin (KLH) was used as a carrier for polypeptide immunizations, the resulting antisera showed a specific immunoreactivity against cell bodies and fibers in layer V of parietal cortex and other scattered locations that was less intense than the specific anti-synthetic polypeptide reactivities discussed hereinbefore. This reactivity was not blocked by the polypeptide, but could be blocked by preincubation with KLH. Other non-brain related anti-synthetic polypeptide sera in whose

-57-

preparation KLH was used as carrier show the same immunoreactivity in brain. Therefore, some cells in brain share an antigenic determinant with KLH.

Antisera to polypeptides coupled to the other two carriers used in this study, edestin and thyroglobulin, also exhibited background reactivities. These reactivities were of a very low level when the antisera were used at dilutions of 1:1000 or greater, and gave light reactions with horseradish peroxidase that were easily distinguishable from the intense, punctate horseradish peroxidase deposits of the anti-synthetic polypeptide-specific reactions.

E. Messenger Ribonucleic Acid

15 (mRNA) Preparation

Cytoplasmic messenger ribonucleic acid (mRNA) was isolated from fresh brains, livers, or kidneys of adult male Sprague-Dawley rats (Charles River) by a phenol/chloroform/isoamyl alcohol (50:50:1) extraction as described in Schibler et al., J. Mol. Biol., 142, 93-116 (1980). The extract was then enriched for polyadenylated mRNA [poly (A)<sup>+</sup> mRNA] by passage over oligo deoxythymidylate (oligo dT) cellulose as described in Aviv et al., Proc. Natl. Acad. Sci. USA, 69, 1408-1412 (1972).

F. Complementary Deoxyribonucleic Acid  
(cDNA) Cloning

cDNA Clones were prepared from brain cytoplasmic poly (A)<sup>+</sup> mRNA by modification of the methods of Wickens et al., J. Biol. Chem., 253, 2483-2495 (1978) and Gough et al., Biochemistry, 19, 2702-2710 (1980). Reverse transcription was carried out for 60 minutes at 42 degrees C. in a volume of 200 microliters under the following conditions: 50 millimolar Tris (2-Amino-2-hydroxymethyl-

1,3-propanediol)-HCl (pH 8.3), 10 millimolar  $MgCl_2$ , 30 millimolar 2-mercaptoethanol, 70 millimolar KCl, 1 millimolar each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP),  
5 deoxyguanosine triphosphate (dGTP), thymidine triphosphate (TTP), 250 microcuries per milliliter  $^{32}P$ -dCTP (800 curies per millimole; New England Nuclear, Boston, Massachusetts), 25 micrograms per milliliter oligo dT, 100 micrograms per milliliter  
10 poly (A)<sup>+</sup> mRNA, and 1000 units per milliliter of reverse transcriptase. Two variations of this technique were used: (1) for preparation 1, the mRNA was pretreated with 2.5 millimolar  $CH_3HgOH$  for 5 minutes at room temperature and actinomycin D (30  
15 micrograms per milliliter) was added to the reaction: (2) for preparation 2, the mRNA was not pretreated and sodium pyrophosphate (4 millimolar) was added to the reaction medium.

After reverse transcription, preparation 1  
20 was first extracted with phenol/chloroform, and then ether. Both cDNA preparations were precipitated several times from the aqueous phases of their reaction media with 0.3 molar sodium acetate and ethanol, dried and resuspended in water. The samples  
25 were heated at 100 degrees C. for 3 minutes and chilled rapidly on ice to dissociate mRNA-cDNA hybrids.

Second strand synthesis was carried out for 3 hours at 15 degrees C. in a reaction volume of 200  
30 microliters containing 50 millimolar HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.2, 70 millimolar KCl, 10 millimolar  $MgCl_2$ , 10 millimolar dithiothreitol, 0.5 millimolar each dATP, dCTP, dGTP, TTP, and 200 units DNA polymerase I,  
35 Klenow fragment [Jackson et al., Eur. J. Biochem.,

45, 623-627 (1974)] (Bethesda Research Laboratories, Inc., Rockville, Maryland). The reaction was stopped by the addition of ethylenediaminetetracetic acid (EDTA) to a 10 millimolar concentration. The double  
5 stranded cDNA (dscDNA) was precipitated with ethanol.

S<sub>1</sub> Nuclease digestion was carried out for 30 minutes at 37 degrees C. in a volume of 200 microliters containing 50 millimolar sodium acetate (pH 4.5), 0.3 molar NaCl, 1 millimolar ZnSO<sub>4</sub>, and  
10 100 units S<sub>1</sub> nuclease (PL Biochemicals, Milwaukee, Wisconsin). After S<sub>1</sub> nuclease digestion, the dscDNA preparations were extracted with phenol/chloroform, then ether, and thereafter precipitated from the aqueous phases with 0.3 molar  
15 sodium acetate and ethanol.

At this stage, the dscDNA preparations were enriched for larger material on a Biogel A-150 m agarose exclusion chromatography beads (Biorad Laboratories, Richmond, California) in a solution  
20 containing 0.15 molar NaCl and 2 millimolar EDTA at pH 8.0, as described in Gough et al., above: preparation 1 was divided into fraction 1A (approximately 1000 to about 1800 base pairs (bp) and 1B (about 500 to about 1000 bp); one fraction, 2A,  
25 (greater than about 500 bp) was derived from preparation 2. Oligo deoxycytidine tails (8 to 15 nucleotides) were added to each dscDNA preparation with terminal deoxynucleotide transferase (PL Biochemicals) 1,000 units per milliliter, in 25  
30 micromolar dGTP, 100 millimolar potassium cacodylate (pH 6.9), 2 millimolar CoCl<sub>2</sub>, 1 millimolar EDTA for 5 minutes at 37 degrees C. as described in Roychoudary et al., Nucleic Acid Res., 3, 865-877 (1976). The tailed dscDNA preparations were  
35 extracted with phenol/chloroform, then ether, and precipitated from the aqueous phases with ethanol.

-60-

Aliliquots of each of the above preparations (10 micrograms per milliliter) were annealed with oilgo deoxyguanosine-tailed, endonuclease Pst I-cleaved plasmid pBR322 (10 micrograms per milliliter), in 100 millimolar NaCl, 10 millimolar Tris-HCl, pH 8, 0.5 millimolar EDTA. The mixtures were heated at 67 degrees C. for 10 minutes, 50 degrees C. for 30 minutes and allowed to cool to room temperature overnight. The deoxyguanosine tails of 8 to 15 nucleotides each were added to the cleaved plasmid as described above for deoxycitidine tails.

E. coli C600 cells were transformed with the recombinant plasmid, and transformants were selected on tetracycline (10 micrograms per milliliter) plates. Individual colonies were transferred to duplicate ampicillin (33 micrograms per milliliter) and tetracycline plates. Ampicillin sensitive, tetracycline resistant ( $Amp^S Tet^R$ ) colonies were selected for further study.

The colonies were numbered and identified as to the origin of the dscDNA insert used in each case, i.e. from dscDNA insert preparations 1A, 1B, 2A. Clones identified as "pO" originate from a pilot cloning study using the method of Wickens et al., supra.

$Amp^S Tet^R$  clones were grown as 1 milliliter overnight cultures in 2YT (1.6 percent Bacto tryptone, 1 percent Bacto yeast extract and 0.5 percent NaCl in water; Difco Laboratories, Detroit, Michigan) medium and plasmid DNA was extracted by the method dexcribed in Birnboim et al., Nucleic Acid Res., 7, 1513 (1979). One-quarter of each culture was digested with Pst I (N.E. Biolabs, Beverly, Massachusetts) and fractionated on a 1 percent agarose gel using 50 millimolar Tris-borate, pH 8.3,

-61-

1 millimolar EDTA in parallel with DNA size standards. Clones giving excisable insert bands of 500 bp or larger were selected for further study.

#### G. Northern Blot Analysis

5 Poly (A)<sup>+</sup> mRNA samples (usually 2 micrograms) were fractionated by electrophoresis on 1.5 percent agarose gels in the presence of 1 molar formaldehyde as described in Rave et al., Nucleic  
10 Acids Res., 6, 3559-3567 (1979), and transferred to nitrocellulose as described in Thomas P.S., Proc.  
Natl. Acad. Sci. USA, 77, 5201-5205 (1980). The blots were prehybridized overnight at 42 degrees C. in 50 percent formamide, 0.75 molar NaCl, 25  
15 millimolar PIPES [1, 4-piperazine-bis-(2-ethanesulfonic acid)], pH 6.8, 0.2 percent sodium dodecyl sulfate (SDS), 25 millimolar EDTA, 100 micrograms per milliliter salmon sperm DNA, 100 micrograms per milliliter yeast mRNA and 5X Denhardt's solution as described in Denhardt, D.,  
20 Biochem. Biophys. Res. Comm., 23, 641-646 (1966). The blots were then hybridized overnight with <sup>32</sup>P-labeled probes at 42 degrees C. in the same medium but with 1X Denhardt's solution.

One-quarter or one-eighth of each crude  
25 plasma extract or (for followup screening) 100 nanograms of purified super-coiled plasmid were labeled with <sup>32</sup>P by nick translation as described in Rigby et al., J. Mol. Biol., 113, 237-251 (1977) to specific activities of 2-4 x 10<sup>8</sup> counts per  
30 minute per microgram. Blots were washed in two changes of 2X SSC standard sodium citrate solution; 30 millimolar trisodium citrate and 0.3 molar sodium chloride at pH 7.0) 0.2 percent SDS for 60 minutes each at 42 degrees C. and once in 0.1X percent SSC  
35 (1.5 millimolar trisodium citrate and 15 millimolar

-62-

sodium chloride at pH 7.0), 0.2 percent SDS for 15 minutes at 67 degrees C. The washed blots were then exposed to Kodak XRP-5 or XAR-1 X-ray film at minus 50 degrees C. using Cronex Lightening Plus intensification screens for 1 or 14 days. Size estimates were based on comparisons with plasmid pBR322 standards as described in Sutcliffe, J. G., Nucleic Acids Res., 5, 2721-2728 (1978).

#### H. Southern Blot Analysis

DNA was extracted from rat liver nuclei after treatment with proteinase K and SDS, digested with Hind III or Eco RI restriction endonucleases, and fractionated on 0.6 percent agarose gels. The DNA was transferred to nitrocellulose as described in Southern, E., J. Mol. Biol., 98, 503-517 (1975) and hybridized with <sup>32</sup>P labeled probes as described for Northern blots.

#### I. Western Blot Analysis

The general method described in Towbin et al., Proc. Natl. Acad. Sci. USA, 76, 4350-4354 (1979) was followed. Rat brain homogenates were pelleted by centrifugation at 20,000 times the force of gravity (Xg) for 20 minutes to provide a crude mitochondrial-synaptosomal pellet. Less than about 10 percent of the total immunoreactivity remained in the supernatant after centrifugation.

The pelleted material was then separated electrophoretically on an 8 percent polyacrylamide-SDS gel, followed by electroblotting onto nitrocellulose. The blotted fractions were then admixed with appropriate anti-synthetic polypeptide antibodies, e.g. anti-P5, -P6 or -P7 sera. Visualization of bound antibody was accomplished using goat anti-rabbit IgG conjugated to HRP as the indicating group, using diaminobenzidine and hydrogen peroxide as discussed hereinbefore.

J. mRNA Abundance Measurements

Recombinant plasmid DNA from 11 clones was prepared from chloramphenicol-treated bacterial cultures by the method described in Tanaka et al., supra, and 10 micrograms of purified DNA from each clone were denatured and immobilized on duplicate nitrocellulose filters as described in Melli et al., J. Mol. Biol., 93, 23-38 (1975). Brain cytoplasmic poly (A)<sup>+</sup> mRNA (4 micrograms) was broken with alkali by incubation at 90 degrees C. for 15 minutes in 50 microlites 10 millimolar Tris, pH 9.5, 1 millimolar spermidine, 0.1 millimolar EDTA and end-labeled by incubation at 37 degrees C. for 60 minutes with 6 microliters 0.5 molar Tris (pH 9.5), 0.1 MgCl<sub>2</sub>, 50 millimolar dithiothreitol, 50 percent glycerol, containing 0.5 microcuries <sup>32</sup>P-ATP (2000 curies per millimole) and T4 polynucleotide kinase (4 units) to give a product mRNA population of approximately 100 nucleotides.

The <sup>32</sup>P-mRNA was hybridized to the plasmid DNA, the nitrocellulose filters were washed and treated with pancreatic mRNAase and proteinase K under the same conditions as described in Derman et al., Cell, 23, 731-739 (1981). The filters were counted, and the percent of steady state poly (A)<sup>+</sup> mRNA hybridizing to each clone was calculated using the following formula:

$$\frac{\text{number average of mRNA size}}{\text{cDNA insert size}} \times \frac{(\text{hybrid-background}) \text{cpm}}{\text{total cpm}} \times 1.5 \times 100$$

The number average size (1760 nucleotides) was used to obtain a number percent rather than a weight percent. The factor 1.5 was used because the poly(A)<sup>+</sup> mRNA preparation was judged to be contaminated by about 30 percent ribosomal mRNA.



The calculated abundance was rounded off into one of the abundance classes; 3.0, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01 percents. The abundances of the other clones was estimated by comparison with these 11 standards, using probe-specific activity, autoradiograph exposure times and band intensity as bases for estimates.

#### K. High Pressure Liquid Chromatography

Analyses by high pressure liquid chromatography (HPLC) were carried out using a Waters Associates Model 204 liquid chromatograph containing a PAC I-125 column (Waters Associates, Boston, Massachusetts) generally following the techniques outlined in Rivier, J. Chromatogr., 202, 211-222 (1980). Elution was carried out in an aqueous buffer containing 0.25 molar triethylamine phosphate (pH 2.25) and 30 percent acetonitrile.

Samples applied in HPLC contained 100 microliters of a 1 molar acetic acid extract of rat brain (4 brains/milliliter). The fractions obtained were analyzed for the formation of an immune reaction with anti-P5, -P6 and -P7 activity by the use of <sup>125</sup>I-labeled sera.

The assays so performed had a sensitivity of 20-50 picograms of proteinoid derivative. Preliminary studies showed the majority of anti-P5 activity to elute from the column with the retention time of a small polypeptide containing about 15 to about 40 amino acid residues. Synthetic polypeptide P5 had a similar elution time.

The elution pattern obtained showed that several, relatively low molecular weight proteinoid derivatives with anti-P5 reactivity were present, thereby indicating an amino acid residue sequence corresponding substantially to that of synthetic

-65-

polypeptide P5 was present in those eluted fractions that could form an immune reaction with anti-P5 serum. That result thereby indicates the presence of an amino acid residue sequence corresponding substantially to that of synthetic polypeptide P5 may be present in a plurality of the eluted fractions.

L. Intraventricular Administration

Rats were equipped with an intracerebroventricular cannula aimed above the lateral ventricle for intraventricular administrations of the synthetic polypeptides. The general procedure of Koob et al., Regulatory Peptides, 2, 153-163 (1981) was followed.

For this surgery, rats were anesthetized with chloropent anesthesia (Fort Dodge Laboratories, Inc.) and secured in a Kopf stereotaxic instrument. A guide cannula, made of 23 gauge stainless steel tubing and 7 millimeters long was lowered to within 1 millimeter of the ventricle and anchored to the skull with two stainless steel screws and dental cement. Coordinates were, with the tooth bar 5 millimeters above interaural zero, -0.6 millimeters posterior to bregma, 2.0 millimeters lateral and -3.2 millimeters below skull surface at the point of entry.

For an injection, the dummy stylet was removed and a 30 gauge stainless steel cannula with 30 centimeter of polyethylene 10 tubing attached was inserted through the guide to 1 millimeter beyond the guide tip. One microliter of solution containing 5 micrograms of polypeptide was injected by gravity over a 30 second period by raising the tubing above the head of the rat until flow began. Volume was measured by marks on the polyethylene 10 tubing previously calibrated with a 5-microliter Hamilton syringe.

-66-

Only those rats whose cannulae flowed easily with this technique were used in the study. All experiments were performed using a blind procedure where the person testing the rats was unaware of the subject's treatment.

V. SPECIFIC EMBODIMENTS OF THE INVENTION

A. Synthetic Polypeptides

One embodiment of this invention is a synthetic polypeptide. That synthetic polypeptide corresponds in amino acid residue sequence to at least a portion of the sequence of a naturally occurring proteinoid, and has a molecular weight equal to less than that of the proteinoid. The proteinoid itself contains an amino acid residue sequence that is translated from a messenger RNA (mRNA) present substantially only in brain cells. The synthetic polypeptide when bound to a carrier as a conjugate and introduced into an animal induces the production of antibodies that bind to the naturally occurring proteinoid or a derivative of that proteinoid in an immune reaction.

Exemplary of such synthetic polypeptides are synthetic polypeptides P1, P2 and P5-P10, discussed hereinbefore. That previous discussion illustrated that the sequences of the synthetic polypeptides corresponded substantially to portions of the sequences of the proteinoids translated by the cell from mRNAs that hybridized with clone plA75 or clone plB236, and that the molecular weights of the synthetic polypeptides were substantially less than the proteinoid molecular weights. This is easily seen from the 318 amino acid residue sequence of the clone plB236 reading frame of the proteinoid and the 34-mer and 21-mer polypeptides of synthetic polypeptides P5 and P6, respectively.

-67-

The presence of mRNA that is cellularly translated into a proteinoid has been illustrated by the in vitro mRNA-stimulated proteinoid synthesis using mRNAs from various tissues. The mRNA that is present substantially only in brain cell tissue and is translated into the naturally occurring proteinoid is preferably polyadenylated, and is present in the cytoplasm as compared to the nucleus or a mitochondria.

The messenger RNA that is translated into the naturally occurring proteinoid is preferably an mRNA that is present at a concentration of less than about 2 percent by weight of the brain cytoplasmic messenger mRNA. More preferably, the mRNA translated into the proteinoid is present at less than about 0.2 weight percent, and most preferably at less than about 0.01 weight percent, of the cytoplasmic mRNA of the brain.

Several synthetic polypeptides were bound to carriers such as KLH, edestin and thyroglobulin to form conjugates, and the conjugates so formed were used to induce the production of antibodies in animals. The synthetic polypeptides so used are preferably at least about 6 amino acid residues long and can be of almost any length less than that of the proteinoid. However, it is preferred that the synthetic polypeptide about 10 to about 50 amino acid residues in length. It is also noted that the synthetic polypeptide can be used alone, inter alia, for raising antibodies and therapeutically, although it is preferred to use a conjugate when antibodies are to be prepared.

Binding of the antibodies so raised has been illustrated by the cytoimmunochemical determinations and HPLC-fractionated proteinoid derivative

determinations discussed before. The proteinoid or proteinoid derivative bound by the antibodies so prepared is preferably a proteinoid that is itself neuroactive or is processed in the cell into one or more neuroactive proteinoid derivatives as is the material bound by antibodies to synthetic polypeptides P5 and P6. In addition, it is preferred that the synthetic polypeptide itself be neuroactive, as is synthetic polypeptide P5, or have a modulating effect on neuroactivity as does synthetic polypeptide P8.

Specific synthetic polypeptides of this invention are exemplified by synthetic polypeptides P1, P2 and P5-P10 whose amino acid residue sequences have been described hereinbefore.

#### B. Receptor Molecules

Biologically active receptor molecules constitute another embodiment of this invention. These molecules are antibodies, or idiotype-containing polyamide portions of antibodies, induced or raised to a synthetic polypeptide of this invention or to its conjugate with a carrier. In preferred practice, the receptors are raised to the preferred synthetic polypeptides of this invention.

The receptors are biologically active in that they bind at least with the antigenic synthetic polypeptide when admixed therewith in aqueous solution, at least at physiological pH values and ionic strengths. Preferably, the receptors also bind with the naturally occurring proteinoid or a derivative of that proteinoid under the same conditions when the proteinoid or its derivative are within brain cell tissue, on the surface of such tissue or when brain cell tissue is suspended or dissolved in an aqueous medium. It is more preferred

that the receptors bind at least to the antigenic synthetic polypeptide within a pH value range of about 5 to about 9, and at ionic strengths such as that of distilled water to that of about one molar sodium chloride.

Idiotype-containing polyamide portions of antibodies are the portions of antibodies that bind to an antigen. Such portions include the Fab, Fab', and F(ab')<sub>2</sub> fragments prepared from antibodies by well-known enzymatic cleavage techniques. Inasmuch as antibodies are discussed in the art as being "raised" or "induced", idiotype-containing polyamide portions of antibodies will also be discussed herein as being "raised" or "induced" with the understanding that a subsequent cleavage step is normally required to prepare such materials from antibodies.

The receptor molecules may be polyclonal as is the case for the antibodies discussed hereinbefore, or the receptors may be monoclonal. Techniques for preparing monoclonal antibodies are well known, and monoclonal receptors of this invention may be prepared by using the synthetic polypeptides of this invention, preferably bound to a carrier, as the immunogen as was done by Arnheiter et al., Nature, 294, 278-280 (1981).

Monoclonal antibodies are typically obtained from hybridoma tissue cultures or from ascites fluid obtained from animals into which the hybridoma tissue was introduced. Nevertheless, monoclonal antibodies may be described as being "raised to" or "induced by" the synthetic polypeptides of this invention or their conjugates with a carrier.

Receptors are utilized along with an "indicating group" also sometimes referred to as a "label". The indicating group or label is utilized

-70-

in conjunction with the receptor as a means for determining whether an immune reaction has taken place, and in some instances for determining the extent of such a reaction.

5           The indicating group may be a single atom as in the case of radioactive elements such as iodine 125 or 131, hydrogen 3 or sulfur 35, or NMR-active elements such as fluorine 19 or nitrogen 15. The indicating group may also be a molecule such as a  
10   fluorescent dye like fluoresein, or an enzyme, such as horseradish peroxidase (HRP), or the like.

          The indicating group may be bonded to the receptor as where an antibody is labeled with  $^{125}\text{I}$ . The indicating group may also constitute all  
15   or a portion of a separate molecule or atom that reacts with the receptor molecule such as HRP-linked to goat anti-rabbit antibodies where the antibody receptor was raised in a rabbit, or where a radioactive element such as  $^{125}\text{I}$  is bonded to  
20   protein A obtained from Staphylococcus Aureus.

          Where the principal indicating group is an enzyme such as HRP, additional reagents are required to visualize the fact that an immune reaction has occurred. Such additional reagents for HRP include  
25   hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine, used herein.

          The terms "indicating group" or "label" are used herein to include single atoms and molecules that are linked to the receptor or used separately,  
30   and whether those atoms or molecules are used alone or in conjunction with additional reagents. Such indicating groups or labels are themselves well-known in immunochemistry and constitute a part of this invention only insofar as they are utilized with  
35   otherwise novel receptors, methods and/or systems.

-71-

C. Assay for the Presence of  
Amino Acid Residue Sequences

Another embodiment of this invention relates to an assay for the presence of a naturally occurring amino acid residue sequence of a brain cell  
5 proteinoid. Here, an above-described receptor is raised and harvested. The receptor (antibody or idio-  
type-containing polyamide portion thereof) is then admixed with brain tissue and an indicating  
10 group. The presence of the naturally occurring amino acid residue sequence is ascertained by the formation of an immune reaction as signaled by the indicating group.

This method is applicable to solid phase  
15 systems such as the brain cell tissue sections described before using antibodies raised to synthetic polypeptides P1 and P2. This method is particularly useful for assaying for the presence of neuroactive proteinoids, or their derivatives whose mRNAs are  
20 present in relatively small amounts, as was shown with antibodies induced by the introduction of synthetic polypeptides P5 and P6 into rabbits. It is also useful in liquid media such as homogenized or other brain cell tissue preparations, as was shown  
25 for the HPLC-fractionated proteinoid derivatives.

One particular liquid medium in which a receptor of this invention is useful is cerebrospinal fluid (CSF). The CSF may be utilized alone as an aqueous liquid medium or admixed with water or a  
30 buffer solution diluent.

In a further embodiment of the invention, cerebrospinal fluid from an animal with a suspected brain injury is provided. When a brain injury occurs, such CSF typically contains a proteinoid or  
35 proteinoid derivative released from the injured brain



cell tissue due to the connection between the brain and spinal cord.

An aliquot of the CSF so provided is admixed with an effective amount of a biologically active  
5. receptor and an indicating group, or a panel of a plurality of different receptors and appropriate indicating groups. The admixture is typically incubated, as is known, for a time sufficient to permit an immune reaction to occur. The incubated  
10 admixture is then assayed for the presence of an immune reaction as indicated by the indicating group. The presence of the proteinoid or its derivative is thereby shown and provides evidence of an injury to brain tissue.

15 Where the proteinoid or proteinoid derivative being assayed is a normal constituent of CSF, this technique is used to measure an increase in the amount present. In this embodiment, an increase in the amount of proteinoid or its derivative  
20 indicates an injury to brain tissue or an abnormality in the brain tissue leading to that increase.

Receptors raised to a synthetic polypeptide having an amino acid residue sequence corresponding substantially to the sequence of a proteinoid or  
25 proteinoid derivative translated from an mRNA present substantially only in specific types of brain cell tissues or in cells of tissue located at a known site in the brain are used in one aspect of this embodiment. Utilization of such receptors thereby  
30 permits the user to identify the type of tissue injured or to locate the site or sites of injury to the brain without resort to surgery.

For example, receptors such as the antibodies induced by introduction of synthetic  
35 polypeptide P1 into a rabbit, described above, may be

utilized to detect an injury to neurons and dendritic processes throughout the brain. A known amount of those receptors is admixed with a CSF aliquot that is thought to contain the proteinoid encoded by the DNA of clone plA75, and the admixture is preferably  
5 incubated to permit an immune reaction to take place. Wells of a microtiter plate are coated with a known amount of synthetic polypeptide P1. The admixture is then added to the wells, incubated, and  
10 rinsed. A known amount of goat anti-rabbit HRP-linked antibodies is then added to the microtiter plates along with requisite amounts of hydrogen peroxide and diaminobenzidine. The extent of the ensuing reaction is then used along with appropriate  
15 standards as an assay for the presence and amount of the proteinoid in the CSF, and thereby as an assay to determine whether the anti-P1 reactive neurons and dendritic processes were injured. It can be readily seen that a similar technique can be used for  
20 proteinoids or their derivatives that are anatomically regionalized as an assay for a brain site-specific injury assay.

It is to be understood that the specificity of this method may be broad, so as to permit an assay  
25 for injury to the brain generally, or the specificity may be narrowed to enable one to locate the site of the injury more closely. When broad specificity is desired, receptors raised to a synthetic polypeptide whose amino acid residue sequence corresponds to the  
30 sequence of a widely distributed proteinoid or proteinoid derivative such as is the case for receptors raised to synthetic polypeptides P1 or P2 are used. Narrowed specificity as to tissue-type injury may be obtained using receptors such as those  
35 raised to synthetic polypeptides P5, P6 or P7.

-74-

A diagnostic system, preferably in kit form, comprises yet another embodiment of this invention.

This system is useful for assaying the presence of a naturally occurring amino acid residue sequence of a proteinoid or its derivative in brain cells by the formation of an immune reaction. This system includes at least one package that contains a biologically active receptor of this invention.

An indicating group or label is preferably supplied along with the receptor and may be packaged therewith or packaged separately. Additional reagents such as hydrogen peroxide and diaminobenzidine may also be included in the system when an indicating group such as HRP is utilized. Such materials are readily available in commerce, as are many indicating groups, and need not be supplied along with the diagnostic system. In addition, some reagents such as hydrogen peroxide decompose on standing, or are otherwise short-lived like some radioactive elements, and are better supplied by the end-user.

Each diagnostic system may be designed for assaying the presence of different amino acid residue sequences. Thus, a system utilized for assaying for the presence of a neuroactive proteinoid or a neuroactive derivative thereof may contain anti-synthetic polypeptide P5, anti-synthetic polypeptide P6 or anti-synthetic polypeptide P7 receptors. Similarly, a system for assaying for the presence of large projection neurons such as pyramidal cells may contain anti-synthetic polypeptide P1 or anti-synthetic polypeptide P2 receptors.

#### D. Pharmaceutical Compositions

Several synthetic polypeptides of this invention are neuroactive; i.e., the polypeptides

-75-

alter the activity of brain cells amid their surroundings when administered to these cells. Synthetic polypeptides P5 and P8 were shown hereinbefore to possess a neurotransmitter-like activity (P5) and neuromodulating activity (P8), respectively. Thus, synthetic polypeptide P5 was shown to increase the firing rate of cells of the CA<sub>3</sub> region of the hippocampus when administered iontophoretically and was found to increase the daytime motor activity of rats when given intraventricularly. Consequently, both synthetic polypeptides P5 and P8 have biological, neuroactivity in the brain that can be measured neurphysiologically and/or behaviorally. In addition, synthetic polypeptide P8 was shown to modulate neuroactivity in the presence of glutamate.

A pharmaceutical composition of this invention includes a synthetic polypeptide of this invention along with a physiologically acceptable diluent such as distilled or deionized water, normal saline, Krebs-bicarbonate solution or the like.

The synthetic polypeptides of this invention may be administered directly into the brain, e.g., intraventricularly, or they may be administered into the CSF as by injection. Typical effective dosages range from about 0.10 micrograms/kilogram of body weight to about 100 micrograms/kilogram of body weight.

Pharmaceutical compositions that contain the synthetic polypeptides as a "pro-polypeptide" may also be administered intervenously (i.v.) or subcutaneously (s.c.). The term "pro-polypeptide" as used herein refers to a derivatized form of a synthetic polypeptide of this invention, which after administration to an animal is altered within the

animal's body to release the synthetic polypeptide or a biologically active derivative of the synthetic polypeptide at the therapeutic site or sites, especially the brain.

5           The pro-polypeptide is preferably a lipophilic derivative of the synthetic polypeptide. The synthetic lipophilic polypeptide derivative is capable of passage from the blood stream through the blood-brain barrier and into brain cell tissues to  
10 facilitate i.v. and/or s.c. administration.

          Pro-polypeptides may be prepared using known reagents that diminish or eliminate the ionic charge that is otherwise normally present on the synthetic polypeptide. For example, the synthetic polypeptide  
15 N-terminal amino groups and lysine epsilon-amino groups may be reacted with reduced N-methyl nicotinic acid or the like to change the positive ionic charge normally present on those amines at physiological pH values to amide groups that are neutral in charge.  
20 The nicotinic acid moiety may also be reduced after the amide-forming reaction is completed.

          The reduced form of N-substituted nicotinic acid moiety is more lipophilic than the above amino groups, and has been shown by Bodor, Science, 214,  
25 1370-1372 (1981) to be useful in derivatizing a primary amino group-containing drug model to permit passage of the derivatized model through the blood-brain barrier. Oxidation to a charged species occurs within brain cells, and the resulting charged  
30 drug model derivative remains within the brain wherein it can be processed into the underivatized drug model. The same reaction sequence is thought to occur with the reduced nicotinamide derivatized synthetic polypeptides of this invention.

Amino groups may also be made more lipophilic by amidification reactions with reactive acids containing 1 to about 10 carbon atoms. Exemplary of such acids are formic acid, acetic acid, pivalic acid, benzoic acid and the like. The acids may be made "reactive" by the use of their acid chlorides or anhydrides, by the use of a carbodiimide coupling agent such as 1-ethyl-3-(3-dimethylamino)-propyl carbodiimide, or the like, as is well known.

10       The positive ionic charge on amino groups may also be eliminated by reactions which change those amines into carbamate linkages. Thus, the amino groups may be reacted with ethyl chloroformate to form the ethyl carbamate (N-carbethoxy) derivatives.

15       The negative ionic charge of a carboxy group may be neutralized by the formation of amide or ester linkage to provide a pro-polypeptide that is relatively more lipophilic than the synthetic polypeptide itself.

20       As was noted hereinbefore, several neuroactive polypeptides found in the brain and elsewhere in the body contain carboxy-terminal amido groups. Such unsubstituted carboxy-terminal amido groups ( $-\text{CONH}_2$ ) are preferred herein for providing the carboxy-terminal portions of the pro-polypeptides of this inventions.

25       Mono-and disubstituted carboxy-terminal amides are also useful wherein the amido nitrogen atom is substituted with a lower alkyl group containing 1 to about 4 carbon atoms, a hydroxy-substituted lower alkyl or wherein the amido nitrogen atom forms a five- or six-membered ring that may contain one or more heteroatoms such as oxygen, sulfur or nitrogen.

Stated differently, the carboxyl function of the carboxy-terminal amino acid residue of a pro-polypeptide may be an amide derivative of the formula  $\text{-CONR}^1\text{R}^2$  wherein  $\text{R}^1$  and  $\text{R}^2$  are

5 independently hydrogen,  $\text{C}_1\text{-C}_4$  alkyl,  $\text{C}_1\text{-C}_4$  hydroxyalkyl, or  $\text{R}^1$  and  $\text{R}^2$  taken together along with the amido-nitrogen form a five- or six-membered ring that may contain one or more heteroatoms selected from the group consisting of oxygen, sulfur

10 and nitrogen. Exemplary  $\text{C}_1\text{-C}_4$  alkyl radicals include methyl, ethyl, iso-propyl, sec-butyl and the like. Exemplary  $\text{C}_1\text{-C}_4$  hydroxyalkyl radicals include 2-hydroxyethyl, 2-hydroxypropyl, 3-hydroxypropyl, and the like. Exemplary five- and

15 six-membered ring radicals, including the amido-nitrogen, include pyrazinyl, pyrazolidyl, pyridyl, morpholinyl, thiomorpholinyl, pyrrolidinyl, triazolyl, triazinyl, and the like.

Sugar molecules such as glucose or ribose

20 may be used as the alcohol portion of ester derivatives of the carboxy-terminal amino acid residue or of any other carboxy group-containing amino acid residue. Mono-functional alcohols containing 1 to about 10 carbon atoms such as

25 methanol, ethanol, benzyl alcohol and the like are also useful in forming ester-containing pro-polypeptides. In addition, pivaloyloxymethyl esters and the like can be prepared by reaction of potassium carboxylate salts with chloromethyl

30 pivalate or another, similar chloromethyl ester such as chloromethyl acetate.

The above-described reactions and derivatizing agents are intended to be exemplary of those which may be used. The use of any specific

35 reaction or derivatizing agent is not a part of this

invention except as that reaction or agent is used to provide pro-polypeptides that are more lipophilic than are the synthetic polypeptides of this invention themselves, such that after s.c. or i.v.

5 administration, the pro-polypeptides so prepared pass from the blood stream through the blood-brain barrier and into the brain with greater facility than do those synthetic polypeptides.

10 The foregoing is intended as illustrative of the present invention but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the novel concepts of the invention.

15

20

25

30

35



WHAT IS CLAIMED IS:

1. A synthetic polypeptide substantially corresponding in amino acid residue sequence to at least a portion of the sequence of a naturally occurring proteinoid and having a molecular weight equal to less than that of said proteinoid, said proteinoid containing an amino acid residue sequence that is translated from a messenger RNA present substantially only in brain cells, said synthetic polypeptide, when bound to a carrier as a conjugate and introduced as said conjugate into an animal, inducing the production of antibodies that bind to said naturally occurring proteinoid or a derivative thereof.
2. The synthetic polypeptide of claim 1 wherein said messenger RNA translated into said naturally occurring proteinoid is present at a concentration of less than about 2 percent by weight of said cytoplasmic messenger RNA of the brain.
3. The synthetic polypeptide of claim 1 wherein said messenger RNA translated into said naturally occurring proteinoid is present at a concentration of less than about 0.01 percent by weight of said cytoplasmic messenger RNA of the brain.
4. The synthetic polypeptide of claim 1 wherein said synthetic polypeptide has an amino acid residue sequence, as represented by a formula below, from left to right and in the direction from amino-terminus to carboxy-terminus, said sequence being selected from the group consisting of
  - (a) CIPEGLESYYTEQ
  - (b) RSVSPWMSVLSEE
  - (c) NVTESPSFSAGDNPHVLYSPEFRISGAPDKYESE;
  - (d) LLGLRGEPPELDLSYSHSDLG;
  - (e) PTKDSYTLTEELAEYAEIRVK;

- (f) LGSERRLLGLRGEPPELDLSYSHSDLG;
- (g) LLGLRGEPPELDLSYSHSDL-NH<sub>2</sub>; and
- (h) LGSERRLLGLRGEPPELDLSYSHSDL-NH<sub>2</sub>.

5        5. A biologically active receptor that  
immunologically reacts with brain cell tissue and  
comprising an antibody or an idiotype-containing  
polyamide portion of an antibody raised to a  
synthetic polypeptide of claim 1 or a conjugate of  
that synthetic polypeptide bound to a carrier, said  
10       receptor forming an immune reaction when admixed with  
brain cell tissue including said naturally occurring  
proteinoid or a derivative thereof.

15       6. The receptor of claim 5 wherein said  
proteinoid or proteinoid derivative is located on the  
surface of said specific brain tissue cells.

7. A method of assaying for the presence  
of a naturally occurring amino acid residue sequence  
of a brain cell proteinoid in a sample of brain cell  
tissues that comprises the steps of

20       (a) providing a biologically active  
receptor of claim 5; and

25       (b) assaying for the presence of said  
naturally occurring proteinoid amino acid residue  
sequence by admixing an aliquot from a sample of  
brain tissue with said biologically active receptor  
in the presence of a group that indicates the  
formation of an immune reaction.

30       8. A diagnostic system for assaying the  
presence of a naturally occurring amino acid residue  
sequence present in at least a portion of a  
proteinoid present in brain cells by the formation of  
an immune reaction comprising at least one package  
containing a biologically active receptor of claim 5,  
said receptors forming an immune reaction with a  
35       proteinoid or derivative of a proteinoid containing

said naturally occurring amino acid residue sequence when admixed with brain tissue.

5           9. The diagnostic system of claim 8 further including a group that indicates the formation of an immune reaction.

          10. The diagnostic system of claim 9 wherein said indicating group is packaged separately from said receptor.

10           11. A pharmaceutical composition containing as an active ingredient an effective amount of a synthetic polypeptide of claim 1, said composition also containing a pharmaceutically acceptable diluent.

15           12. The pharmaceutical composition of claim 11 wherein said synthetic polypeptide is present as a lipophilic pro-polypeptide derivative that is capable of passage from the blood stream through the blood-brain barrier and into brain cell tissues.

20           13. The pharmaceutical composition of claim 11 wherein said synthetic polypeptide has an amino acid residue sequence as represented by a formula below, from left to right and in the direction from amino-terminus to carboxy-terminus, said sequence being selected from the group consisting of

- 25           (a) NVTESPSFSAGDNPHVLYSPEFRISGAPDKYESE;  
             (b) LLGLRGEPPELDLSYSHSDLG;  
             (c) LGSERRLLGLRGEPPELDLSYSHSDLG;  
             (d) LLGLRGEPPELDLSYSHSDL-NH<sub>2</sub>; and  
             (e) LGSERRLLGLRGEPPELDLSYSHSDL-NH<sub>2</sub>

30           14. A method for determining whether animal's brain tissue has been injured by the formation of an immune reaction comprising the steps of

- (a) providing cerebrospinal fluid from  
35    an animal suspected of having a brain tissue injury,

-83-

said cerebrospinal fluid containing a proteinoid or proteinoid derivative released from injured brain tissue when an injury to said tissue has occurred;

5 (b) admixing an aliquot of said cerebrospinal fluid with effective amounts of (i) a biologically active receptor of claim 5 and (ii) an indicating group; and

10 (c) assaying said admixture for the presence of an immune reaction between said proteinoid or proteinoid derivative in said cerebrospinal fluid and said receptor, the presence of an immune reaction being indicated by said indicating group, and said immune reaction being indicative of an injury to brain tissue.

15 15. The method of claim 14 wherein said proteinoid or proteinoid derivative is present in neurons and dendritic processes in the brain.

20

25

30

35



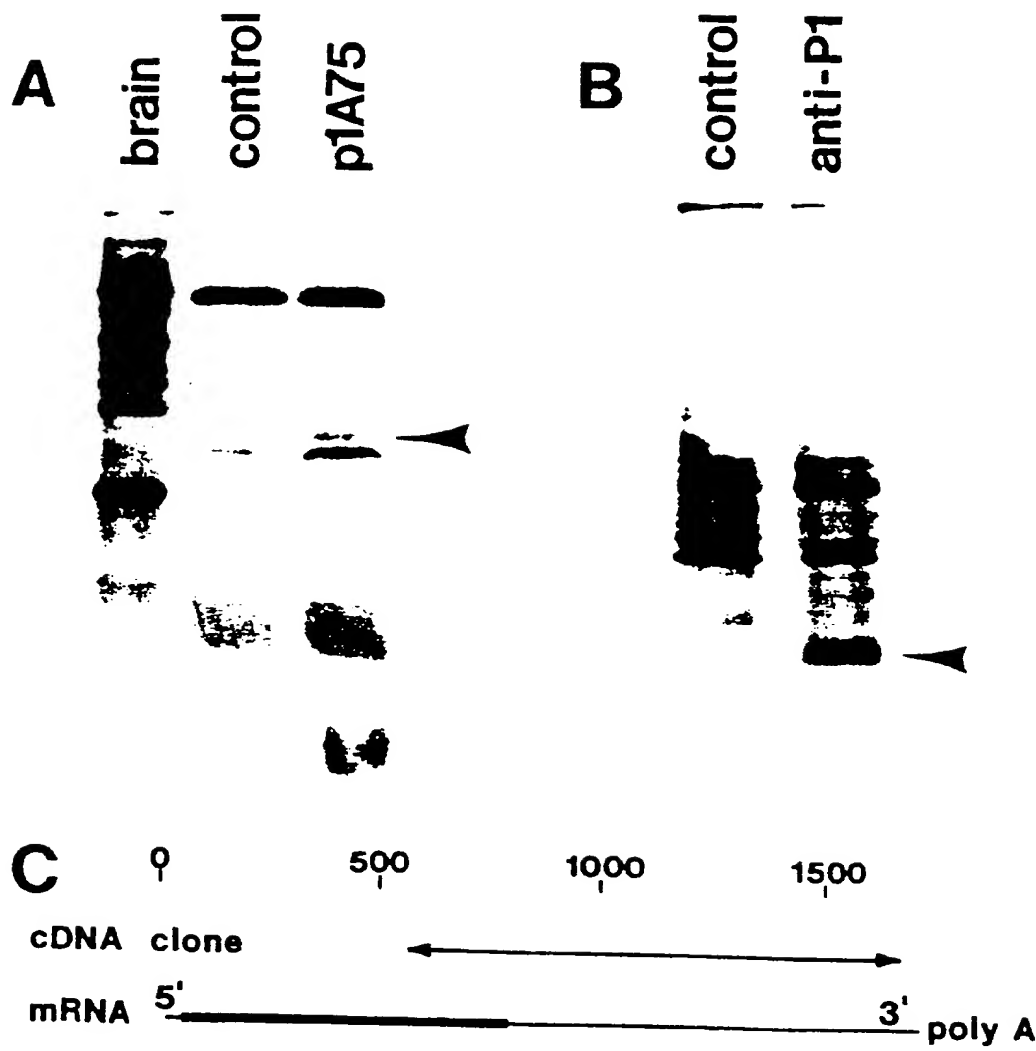


FIGURE 2

FIGURE 3

